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Local mate competition and the sex ratios of malaria parasites, with a focus on *Plasmodium mexicanum*

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LOCAL MATE COMPETITION AND THE SEX RATIOS OF MALARIA
PARASITES, WITH A FOCUS ON *PLASMODIUM MEXICANUM*

A Dissertation Presented

by

Allison Theresa Neal

to

The Faculty of the Graduate College

of

The University of Vermont

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ABSTRACT

Sex ratio theory is a focus in evolutionary biology that explores how natural selection shapes investment in males and females. It has provided some of the best quantitative evidence of evolution and could find utility in public health efforts through its application to malaria parasites. These parasites have distinct male and female forms that are produced following massive asexual replication, and they mate within the blood-feeding insects that transmit them between vertebrate hosts. A very similar population structure is assumed by local mate competition (LMC), a model from sex ratio theory that predicts female-biased sex ratios dependent on the degree of selfing within a mating patch.

In this dissertation, I test a series of predictions from LMC for the lizard malaria parasite *Plasmodium mexicanum*. These include: (i) sex ratios have heritable variation that is not constrained by other life history traits; (ii) single-genotype infections have female-biased sex ratios that are determined by male fecundity; (iii) multiple-genotype infections have less biased sex ratios than single genotype infections; (iv) if males are limiting, sex ratios may be less biased when there are fewer parasites present (an extension of LMC called fertility insurance); and (v) less biased sex ratios may also be favored if increased female production yields diminishing returns on transmission to a new vertebrate host. To test these predictions, I combined the study of natural and experimental infections, microscopy (parasite density and sex ratio), molecular genetics (infection genetic diversity), and mathematical modeling (of how transmission patterns might affect sex ratio evolution).

Overall, the results were qualitatively consistent with both LMC and my new model predictions. Sex ratios showed evidence of heritable variation that was unlinked to other life history traits measured. Sex ratios in single-genotype infections were female biased and consistent with the male fecundity observed, and were lower than sex ratios in experimental multiple-genotype infections, as predicted. Sex ratios were not less biased with lower sexual cell density, suggesting that males were not limiting. In fact, the opposite trend was sometimes observed: sex ratios were less biased with more sexual cells. This pattern has been observed previously in this and other species, and the only model that currently predicts such a trend is the new transmission model I outline.

This dissertation contributes to our understanding of sex ratio evolution for malaria parasites in a number of ways. First, it adds evidence to the idea that the selective forces implicated in LMC are at work in malaria parasites and that malaria parasites are able to detect and respond to relevant cues. Second, it helps account for discrepancies in existing data, which have often reached conflicting conclusions. Third, it offers one of the first detailed studies of malaria parasite male fecundity, an essential piece of the sex ratio puzzle. Finally, it outlines a new theoretical extension of LMC that provides novel predictions and highlights areas of study that may be fruitful for future work on malaria parasites and other organisms.

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CHAPTER 1: Introduction and Literature Review

Allison T. Neal

Sex Allocation Theory: An Overview

The study of how selection shapes relative investment in male and female offspring or function has often been celebrated as one of the most successful and productive foci in evolutionary biology (West 2009, West and Herre 2002, Pen and Weissing 2002, Edwards 1998). This degree of success is owed in part to the simplicity and specificity of the trait being studied (Orzack 2002), especially for sexually dioecious organisms; individuals are either male or female, and the ratio of one sex to the other can often be modeled quantitatively and measured with a great degree of precision (West and Herre 2002, West 2009). Indeed, sex allocation theory has benefited greatly from a sustained interchange between theoretical models and empirical tests of those models (Bull and Charnov 1988, West 2009 provides multiple examples). However, its distinction is not due to its well-supported status alone. Sex allocation theory has also provided a testing ground for some debates that have been central to the study of evolutionary biology in general. It has allowed assessment of group selection theory, in both its outdated “good of the species” form (Sober 2001, Williams 1966) and the more modern balancing of interdemic and intrademic selective pressures (West 2009). Sex allocation theory also employed some of the first evolutionary models from game theory (Maynard Smith 1982), and has displayed the utility of such models, which consider phenotype without the complication of underlying genetic architecture (West 2009). Furthermore, this research focus provides well-supported examples of many phenomena

like frequency dependent selection (Charnov 1982), genetic conflict (West and Herre 2002), and phenotypic plasticity (West and Herre 2002).

The study of sex allocation originated with Darwin, who recognized that population-wide sex ratios could be shaped by selection on individuals (Edwards 1998). Düring (1884, translation in Edwards 2000) developed the first mathematical model (which was, in fact, ‘perhaps the first mathematical model in evolutionary biology’; Seger and Stubblefield 2002) and the idea of sex ratio evolution was popularized by Fisher (1930), who showed verbally that frequency dependent selection favoring the less common sex would lead to the evolution of populations with equal investment in the two sexes. This is because, as Fisher emphasized, every individual has one father and one mother. The total genetic input of a generation, therefore, comes equally from the females (collectively) and the males (collectively) of the previous generation. If there are fewer of one sex than the other in a population, this total genetic input to the next generation will be split among fewer individuals, thus giving a higher per capita fitness to that sex. Mothers that produce the less common sex gain a fitness advantage via genetic contribution to more grand offspring, and may pass that tendency on to their offspring, thereby shifting the population sex ratio toward parity.

This model makes a number of simplifying assumptions that are not met by many organisms, and the field expanded as subsequent models incorporated different aspects of organisms’ distribution, ecology, and genetics. The first of these new models were proposed by Hamilton (1967) and overturned the implicit assumptions of random population-wide mating and control of sex allocation by genes with Mendelian inheritance. This paper has been cited as one of the greatest in evolutionary biology

(Orzack 2002) and highlights how sex-biased competition within species and genetic conflict could select for unequal sex ratios. In doing so, the paper laid the foundation for many of the subsequent theoretical advances (Bull and Charnov 1988), most of which can broadly be grouped into three categories: models that focus on sex-biased competition or cooperation, models based on the condition or circumstances of mothers, and models that incorporate conflict between genes or individuals.

For many species, cooperation or competition between related individuals may be stronger for one sex than the other. For example, if males disperse and females do not, there may be more competition between related females for resources than between males. Alternatively, daughters may remain with their mothers for a period and help care for their brothers and sisters, thereby enhancing the reproductive potential of their mother. The models from sex allocation theory that incorporate such interactions, local resource competition (first proposed by Clark 1978) and local resource enhancement (first proposed by Trivers and Willard 1973 in a footnote), predict an overproduction of whichever sex competes less with related individuals or enhances their fitness most. This group of models includes local mate competition, which was presented in Hamilton's (1967) pioneering paper and is discussed in more detail below.

A second type of model relates generally to parental condition or circumstances. Generally referred to as the Trivers and Willard hypothesis (after their 1973 paper that first presented the idea), the model is based on the idea that one sex may benefit more from being in good condition than the other, such as males of species in which there is intense competition for access to females (e.g. control of harems) or animals for which clutch size in females is more resource dependent than mating opportunities for males. In

such a case, parents (generally mothers) that are in better condition or have better circumstances are expected to produce offspring that are also in better condition, and should therefore produce more of whichever sex will benefit more from this improved condition (Trivers and Willard 1973). While originally applied specifically to mammals, this model is relevant to a variety of biological systems, such as parasitoid wasps that lay their eggs in hosts of different sizes (larger hosts lead to higher quality offspring), mate attractiveness in birds and lizards (more attractive mates may lead to more attractive offspring), environmental sex determination (can bias sexes produced at different times of year when offspring will be higher/lower quality), and the timing and order of sex change in sequential hermaphrodites (West 2009). Such sex ratio shifts are on the level of the individual and may occur even in populations in which the sex ratio overall is unbiased; in this case, biases toward one sex in good condition parents will be balanced by biases toward the other sex for those in poorer condition.

The final group of models are those that deal with conflict at multiple levels. Many models considering the evolution of sex allocation, including 'Fisher's' model, assume it is a trait that shows Mendelian inheritance and control by one parent (or that the parents 'agree' on which sex ratio is best; Bull and Charnov 1988, West 2009). However, there are many situations in which these assumptions do not apply. For example, sex allocation may be controlled either by genes on a sex chromosome or by cytoplasmic elements (including endosymbionts like *Wolbachia*). In this case, these genes would be inherited only through one sex, greatly altering the sex ratio that grants the greatest fitness benefit to these genes relative to genes with Mendelian inheritance (Hamilton 1967, West 2009). While many examples of this type of conflict involve

cytoplasmic elements and favor a female bias, it is also possible that the genes controlling sex allocation are located on the Y-chromosome of organisms in which males are the heterogametic sex, and could therefore also favor a male bias (Hamilton 1967).

Conflict can also operate at the level of individuals. Parents and offspring often do not agree on the optimal sex allocation strategy. This conflict has received particular attention in the social haplodiploid hymenoptera (West 2009), where queens and workers often do not agree on the optimal sex ratio of the colony due to issues of relatedness (Hamilton 1972). Queens are equally related to their sons and daughters, but workers are more closely related to full sisters than to brothers, and more closely related to their own sons (if they are not sterile) than to half sisters (if queens have mated multiply). Conflict over optimal sex allocation can therefore arise between queens and workers (e.g. Herbers 1984), with the specific direction of the bias preferred by each party dependent on the number of males a queen has mated with and whether or not workers are sterile. Also, while most empirical work on individual-level conflict has focused on haplodiploid systems, Trivers (1974) showed that conflict between mothers and fathers and between mothers and offspring could also arise in diploid systems if male and female offspring require different amounts of energy invested. In the absence of other selective pressures, mothers should invest equal amounts of energy in each sex (meaning that she would produce fewer of the more expensive sex), but selection on offspring and fathers (if mothers mate multiply) favors greater investment in the numerically less abundant sex due to its greater per capita fitness (Trivers 1974).

This is by no means a comprehensive review of all models in sex ratio theory. Many other models and model variations exist, including those with explicit genetic

mechanisms, overlapping generations, different sexual systems (e.g. gynodioecy, hermaphroditism, haplodiploidy, etc.) and limitations on environmental predictability, information availability, and fecundity (male and female). My goal here was not to provide a list of all models of sex ratio evolution that have been conceived or tested, a pursuit to which others have devoted entire books (e.g. West 2009), but rather to provide a brief overview the variety of biological circumstances to which sex allocation models have been applied and then to focus on the models that are most relevant to research presented in this dissertation, specifically local mate competition and one extension of this model, fertility insurance.

Local Mate Competition and Fertility Insurance

In an already distinguished field of research, local mate competition holds a particularly prominent position. Local mate competition has provided some of the best quantitative support in sex allocation theory (West et al. 2001, West 2009), and it is the model responsible for elucidating many of the general themes from evolutionary biology highlighted above, including the comparison of group selection vs. inclusive fitness models, the use of game theory in evolutionary biology, and the evolution of phenotypic plasticity.

Local mate competition (LMC) is a special case of local resource competition and local resource enhancement in which female-biased sex ratios both reduce competition among related males for access to females and enhance the reproductive success of sons due to the excess of daughters (Taylor 1981). The selective advantage of the female-biased sex ratios predicted by LMC results from the assumed structuring of the population. This model proposes a population for which suitable habitat is divided into

patches. Each of these patches is colonized by a small number of mated females, who each deposit an equal, large number of offspring in the patch. The offspring mature in the patch and mate exclusively with other members of the patch before mated females disperse to new patches.

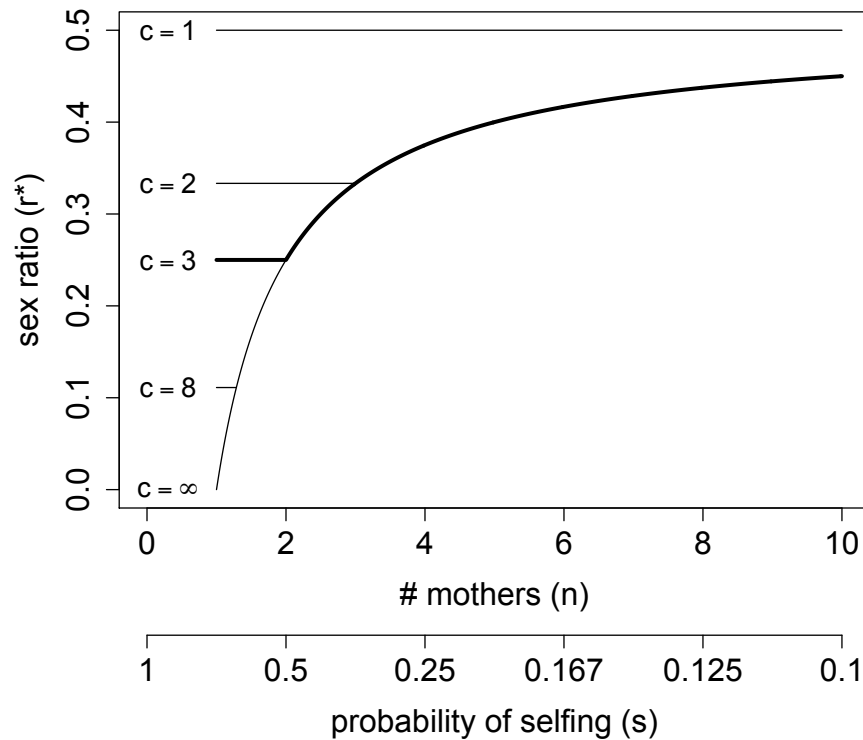


Figure 1 Sex ratios predicted by the local mate competition model from sex ratio theory. Mathematical expressions describing these relationships are given in the text. The bold line emphasizes the shape of the relationship if male fecundity is low ($c = 3$).

As hinted at above, one of the particular sources of this model's success is its ability to quantitatively predict sex ratios based on only one parameter: the number of mother's depositing offspring on each patch (n). The sex ratios predicted by this model are highly female biased when only a single mother deposits offspring in a patch- in fact, under these conditions the mother should produce just enough sons to mate with all of her daughters (Hamilton 1967). The sex ratios, generally expressed as proportion male, increase to an asymptote of 0.5 in patches with increasing numbers of mothers. This

relationship is described by the equation $(n - 1)/2n$ and shown in Figure 1. A lower limit is placed on this relationship to ensure that enough males are produced to mate with all females. This lower bound is also shown in Figure 1 and is described by the equation $1/(1 + c)$, where c is the number of females each male can mate with (male fecundity; Hamilton 1967).

Different authors have modeled LMC using different methods and perspectives, and encouragingly all come to the same conclusions. Hamilton himself presented three different methods of considering LMC in his original (1967) paper, combining a mathematical derivation based on maximizing individual fitness, a computer simulation to identify the fittest of a set of genotypes, and a game theory approach identifying unbeatable and unexploitable sex ratios that could be ‘played’ against an opponent based on the opponent’s strategy. Other approaches include group selection models (e.g. Colwell 1981, Wilson and Colwell 1981) and models with explicit genetic mechanisms (e.g. Taylor and Bulmer 1980). While multiple methods of modeling sex ratio evolution with LMC yield the same sex ratio predictions, West (2009) argues that not all are equally useful. Most extensions of LMC are based on an inclusive fitness rather than group selection framework, highlighting the fact that this type of model is easier to formulate and more general. Similarly, models considering sex ratio only as a phenotype without incorporating the underlying genetic mechanisms are simpler than genetic models, and their utility is highlighted by the consistency of their predictions with those of genetic models and empirical data.

In addition to these different approaches to LMC, many researchers have extended the theory by altering certain key assumptions, just as LMC extended

‘Fisherian’ sex ratio theory by altering the assumption of a randomly mating panmictic population. These include extensions altering the assumptions of an infinite number of patches (Taylor and Bulmer 1980), a single generation per patch (Bulmer and Taylor 1980), equal offspring production by all mothers (Yamaguchi 1985, Stubblefield and Seger 1990), complete knowledge of patch conditions (e.g. n , offspring production of other mothers; Stubblefield and Seger 1990), and others (summarized in West 2009).

One extension of LMC that is of particular importance to the research outlined in this dissertation is Fertility Insurance. This extension of LMC explores the consequences of factors that reduce the chances of there being sufficient males to ensure fertilization of all females and shows that less biased sex ratios than would be expected under standard LMC are favored. The extension was originally proposed in reference to organisms with a population structure similar to LMC but for which not all offspring in a patch survive to mate (Hartl 1971, West 2009). If males are very rare, there is an appreciable probability that no males will survive to adulthood, meaning that the females will not be mated. Producing a less biased sex ratio decreases the chance that no males will reach maturity. This LMC extension has received considerable recent attention in reference to sex ratio evolution in malaria parasites and other apicomplexans (West et al. 2001, 2002, Garnder et al. 2003). As will be discussed in more detail below, these organisms have a population structure that closely approximates the assumptions of LMC (Read et al. 1992), but a number of traits and conditions they experience could limit male success or availability including low male fecundity, low population density or mobility within a patch, and high mortality within a patch (West et al. 2001).

Malaria Parasite Sex Ratios

Local Mate Competition has been tested largely with multicellular organisms (Read et al. 2002), particularly insects with a haplodiploid genetic system (Pen and Weissing 2002). However, within the past 25 years, researchers have begun to investigate the sex ratios of sexually reproducing, anisogamous single-celled organisms such as the parasites that cause malaria (*Plasmodium* and related genera sensu Martinsen et al. 2008; Schall 1989, 2009, Read et al. 1992, 2002). In addition to providing taxonomic diversity to the empirical data testing LMC, multiple authors have highlighted the potential applications that could come from a better understanding of sex ratio evolution in these medically important organisms. For example, sex ratios may provide a cheap and efficient way of estimating inbreeding rates and genetic diversity in populations of malaria parasites, which may also be linked to virulence and the evolution of drug resistance (Read et al. 1992, West 2009). Discrepancies between empirical results and theoretical predictions as well as unexplained variation may help identify species for which our understanding of their basic biology is poor, and thereby highlight areas of future research (Read et al. 2002, Shutler and Read 1998, Schall 2009). Research on sex ratios may even help identify the cues used by parasites to determine sex allocation, which could be manipulated to produce maladaptive sex ratios that might reduce transmission (Schall 2009).

Another factor making the study of malaria parasite sex ratios worthwhile is the substantial variation that exists both among and within species (Table 1). Observed sex ratios range from strongly female biased (e.g. Burkot et al. 1984 report cultures of *Plasmodium falciparum* with only 2.5 – 10% males) to almost equal numbers of males

and females (e.g. *Plasmodium mexicanum* generally has sex ratios around 40-45% male; Osgood et al. 2002, 2003, Osgood and Schall 2004, Schall 1989, 2000), with a few reports (all in one paper) of strangely male-biased sex ratios (various *Haemoproteus* of lizards; Paperna and Landau 1991).

Table 1 Summary of existing sex ratio data (expressed as proportion male) showing variation among and within species. The data provided differed among papers, so the sex ratio column contains either some kind of average (typically mean or median) or a range of sex ratios observed across groups of infections (e.g. genotypes), individual infections or infections over time. Some studies differentiated between sex ratios early (e) or late (l) in the course of infection, or between infections with a single (s) or multiple (m) clones. The tilde (~) indicates sex ratios were estimated, e.g. from a figure. Also included, if indicated, are the number of infections sampled (#inf) and the number of gametocytes per count that was made. Note that some studies sampled clones or isolates, potentially from multiple different cultures. These are indicated listed under #inf with a (c).

species	sex ratio	#inf	gam/count	Ref.
<i>Plasmodium agamae</i>	0.401	30	100	Schall 1989
<i>Plasmodium balli</i>	0.26	22	1-200	Pickering et al. 2000
<i>Plasmodium cathemerium</i>	0.48	8		Talliaferro 1925 (cited by Schall 1989)
<i>Plasmodium chabaudi</i>	e: ~0.18 l: ~0.30	56	52.26 (av.)	Reece et al. 2003
<i>Plasmodium chabaudi</i>	s: ~0.15 m: ~0.45	40	lots (PCR)	Reece et al. 2008
<i>Plasmodium falciparum</i>	0.064	5 (c)	100	Burkot et al. 1984
<i>Plasmodium falciparum</i>	0.29	1	>368	James 1931
<i>Plasmodium falciparum</i>	0.46	95	-	Mitri et al. 2009
<i>Plasmodium falciparum</i>	0.16	23	100+	Read et al. 1992
<i>Plasmodium falciparum</i>	0.217	54	>15	Robert et al. 1996
<i>Plasmodium falciparum</i>	0.278	284	>10	Robert et al. 2003
<i>Plasmodium falciparum</i>	0.2	11	100	Smalley and Sinden 1977
<i>Plasmodium falciparum</i>	0.22	162	36 (av.)	Sowunmi et al. 2009a and b
<i>Plasmodium falciparum</i>	~0.5	3 (c)	-	Trager et al. 1981
<i>Plasmodium falciparum</i>	≤0.2	3 (c)	-	Carter and Miller 1979
<i>Plasmodium falciparum</i>	0.172	-	-	Boudin et al. 1993
<i>Plasmodium vivax</i>				
<i>Plasmodium gallinaceum</i>	e: 0.1-0.2 l: ~0.5	-	100 or 50,000RBC	Paul et al. 1999, Paul 2000
<i>Plasmodium giganteum</i>	0.371	30	100	Schall 1989
<i>Plasmodium mexicanum</i>	0.40	150	100	Osgood and Schall 2004
<i>Plasmodium mexicanum</i>	~0.40-0.45	109	100 (86%)	Osgood et al. 2002
<i>Plasmodium mexicanum</i>	~0.35	70	100	Osgood et al. 2003
<i>Plasmodium mexicanum</i>	0.372	63	>50	Schall 2000
<i>Plasmodium mexicanum</i>	0.474	54	100	Schall 1989
<i>Plasmodium nucleophilum</i>	0.57	-	-	Manwell and Voter 1939 (cited by Schall 1989)

<i>Plasmodium 'tropicum'</i>	0.32	36	1 to 200	Pickering et al. 2000
<i>Plasmodium ovale</i>	0.11-0.38	2	-	James et al. 1932 (cited by Schall 1989)
<i>Plasmodium vinckei</i>	e: 0.5-0.10 l: ~0.4-0.5	4	-	Paul 2000
<i>Plasmodium vivax</i>	0.27	≥15	-	Zollner et al. 2006
<i>Haemoproteus cf. opluri</i>	0.08-0.28	-	-	Paperna and Landau 1991
<i>Haemoproteus endomensis</i>	0.025-0.43	2	-	Paperna and Landau 1991
<i>Haemoproteus gehyrai</i>	0.58-0.92	1	-	Paperna and Landau 1991
<i>Haemoproteus mackerrasi</i>	0.58	1	-	Paperna and Landau 1991
<i>Haemoproteus majoris</i>	0.47-0.54	64	100	Merino et al. 2004
<i>Haemoproteus oedurae</i>	0.57-0.94	3	-	Paperna and Landau 1991
<i>Haemoproteus opluri</i>	0.52-0.85	3	-	Paperna and Landau 1991
<i>Haemoproteus pyodactyli</i>	0.64-0.9	1	-	Paperna and Landau 1991
<i>Haemoproteus simondi</i>	0.32	2	-	Paperna and Landau 1991
<i>Haemoproteus tarentolae</i>	0.42	4	-	Paperna and Landau 1991
<i>Haemoproteus underwoodsauri</i>	0.5-0.57	1	-	Paperna and Landau 1991
<i>Haemoproteus spp.</i>	~0.37	~234	100+	Shutler et al 1995
<i>Leucocytozoon spp.</i>	~0.2-0.45	202	87 (av.)	Read et al. 1995

Schall (1989) first introduced sex ratio theory to the study of malaria parasites, and Read et al. (1992) proposed that the assumed population structure of LMC fits the life history of malaria parasites well. For these organisms, suitable habitat consists of separate hosts, the patches of LMC. Each malaria parasite species has two hosts: a vertebrate and a blood-feeding insect (Fig. 2). A vertebrate becomes infected when an infectious insect bites it and sporozoites, the haploid transmission stages, are transferred in the insect's saliva. The parasite then undergoes many cycles of asexual replication, first in fixed tissues like the liver and then in host blood cells (Fig. 2, 1). Some cells leave the cycle of asexual replication and differentiate into male and female sexual cells, the gametocytes (Fig. 2, 2). The specific processes that govern sexual differentiation and the switch from asexual reproduction to gametocytogenesis are still largely unknown, but it is well accepted that the haploid male and female cells are genetically identical but differ in protein expression (reviewed in Baker 2010, Liu et al. 2011). Male and female

gametocytes undergo no further development or replication unless they are ingested by their insect host. Upon reaching the insect's midgut, they form gametes and mate within minutes (Fig. 2, 3). Males release up to 8 flagellated gametes and females develop into one large gamete. The resulting zygote is diploid (Fig. 2, 4), but undergoes meiosis followed by further asexual replication to produce the haploid sporozoites (Fig. 2, 5), which travel to the insect's salivary glands to be transmitted (Fig. 2, 6). Malaria infections generally contain only one or a few asexually derived clonal lineages (Paul and Day 1998, Read and Day 1992), which are analogous to the offspring deposited in patches by one or a few mothers in the LMC model.

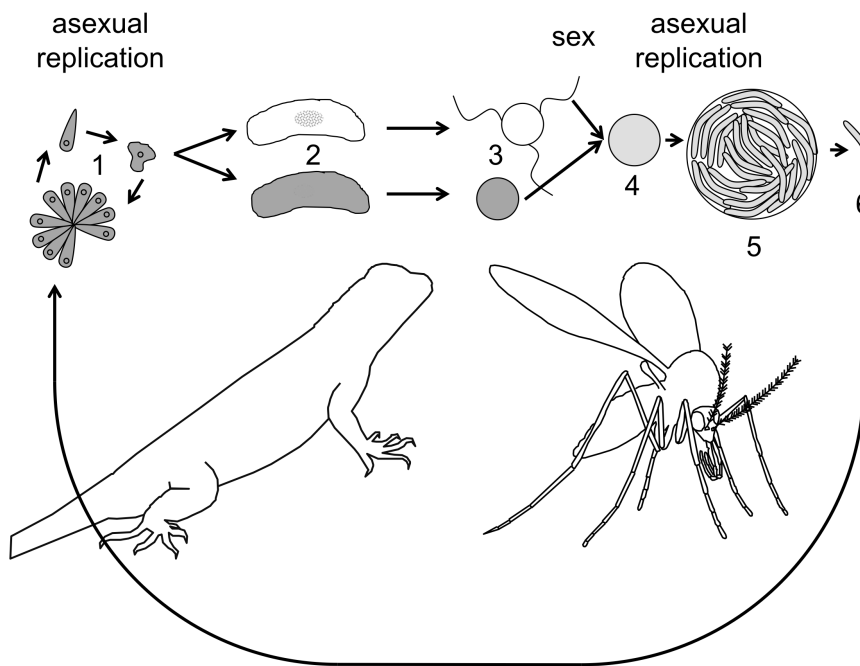


Figure 2: Life cycle of a malaria parasite. Descriptions corresponding to each number in the figure are given in the text. The hosts depicted are for the lizard malaria parasite *Plasmodium mexicanum* but the life cycle is common to all malaria parasites.

A few empirical tests of LMC predictions for malaria parasites have provided strong support for this model, but many have been less certain. Perhaps the best support for LMC comes from a study of experimental infections of the rodent malaria parasite *Plasmodium chabaudi*. To test whether this parasite facultatively adjusts its sex ratio in response to the presence of other clones, Reece et al. (2008) compared the sex ratios produced by clones individually with those produced in infections with multiple of the same clones combined (two or six). The resulting sex ratios closely match both the qualitative and quantitative predictions of LMC (Reece et al. 2008; Schall 2009). These results suggest that the parasite is able to detect and respond to co-infecting clones and are particularly striking given that the experiments were performed using white lab mice as hosts, rather than the parasite's natural host, the thick-ket rat *Thamnomys rutilans* (Schall 2009). Sowunmi et al. (2009b) similarly compared clone numbers with sex ratio, but for natural human infections of *Plasmodium falciparum*. They also found a good qualitative match with theory for infections with one to three clones, but sex ratios in infections with four and greater than four clones did not differ significantly from those with three, nor did they appear to differ from those with two (comparison not made statistically, but see Fig. 2 in Sowunmi et al. 2009b).

Indirect estimates of clone numbers have also been used to predict qualitative trends in sex ratio across infections. For example, Osgood and Schall (2004) compared sex ratios for experimental infections of the lizard malaria parasite *Plasmodium mexicanum* initiated from either one donor infection or three donors combined. They assumed that infections from three donors would have more clones than those with one, and therefore predicted that sex ratios would be more female-biased in infections with

one donor than in those with three. No difference in the sex ratios of these two groups was observed. Pickering et al. (2000) took a different approach, and assumed that the density of sexual cells was positively correlated with the number of clones, which would predict a positive correlation between gametocyte density and sex ratio. They tested this prediction for two species of lizard malaria parasite, *Plasmodium 'tropicum'* and *Plasmodium balli*. The predicted pattern was observed for the former species but not for the latter. They suggest that the data from *P. 'tropicum'* show support for the LMC model, but that other explanations are certainly possible because they are observational data (Pickering et al. 2000). Chapter 7 of this dissertation provides one alternative explanation.

In addition to comparisons of sex ratios among infections, some researchers have used population sex ratios to test the predictions of LMC. Read et al. (1992) derived an expression relating sex ratio to the probability of mating with a genetically identical individual, or selfing (s , which is equivalent to the inbreeding coefficient F ; Dye and Godfray 1993): $r^* = (1 - s)/2$. They used this expression to predict the degree of inbreeding for *P. falciparum* in Papua New Guinea based on the observed sex ratios of infections in that region. The predicted inbreeding coefficient was consistent with a later estimate based on molecular data (Paul et al. 1995), but only if it is assumed that non-amplifying samples are not due to the presence of null alleles (Anderson et al. 2000).

Population-wide measures of sex ratio have also been used for among-population comparisons. Read et al. (1995) showed that the proportion of infected hosts in a population ('parasite prevalence') could be used to estimate the average selfing rate provided that parasites are dispersed among hosts following a negative binomial

distribution (Anderson and May 1991). Thus, a predicted relationship between prevalence and sex ratio could be established. Read et al. (1995) found good empirical evidence that such a relationship exists for populations of *Leucocytozoon* spp., but Shutler et al. (1995) failed to find a similar relationship for populations of *Haemoproteus* spp.

A potential explanation for the discrepancy between the *Leucocytozoon* and *Haemoproteus* results arises from Fertility Insurance (Shutler and Read 1998). Fertility Insurance, as described in the previous section, predicts less biased sex ratios when there is a higher probability that there will not be sufficient males to ensure fertilization of all females. *Haemoproteus* have smaller insect vectors than *Leucocytozoon*, and small vectors may limit the total number of gametocytes consumed, favoring a higher production of males overall to boost fertilization rates. This selective pressure could override the effect of variable selfing rates among populations by shifting the minimum predicted sex ratios above those predicted by standard LMC.

Currently, the empirical data supporting Fertility Insurance seems more convincing than the evidence of standard LMC. Evidence of increased investment in males in response anemia (or associated cues, such as induction of erythropoiesis) and low gametocyte density has been recorded from multiple species (*Plasmodium gallinaceum* Paul 2000; *Plasmodium vinckei* Paul 2000; *P. chabaudi* Reece et al. 2008; *P. falciparum* Robert et al. 2003, Sowunmi et al. 2009a; *Haemoproteus majoris* Merino et al. 2004). Anemia and low gametocyte density can decrease both the total number of gametocytes consumed by an insect vector (i.e. the mating population size) and the number of gametocytes that are physically able to interact during the short period

available for mating (i.e. the mating group size, Gardner et al. 2003, West et al. 2001, Paul et al. 2002). Sex ratios have also been observed to become less female-biased over the course of infections (*P. falciparum* James 1931), particularly for infections in which a successful immune response is mounted (*P. gallinaceum* Paul et al. 1999), which could indicate a facultative increase in male production in response to increased immune-related mortality. A few studies have failed to support the predictions of LMC, but most so far relate to one species: *P. mexicanum*. For this species, there was no observed effect of testosterone treatment (predicted to down-regulate immune response and therefore remove selection for less-biased ratios; Osgood et al. 2003), no consistent increase in sex ratio over time (Osgood and Schall 2004, Osgood et al. 2002), and either no relationship with gametocytemia or a positive one, contrary to Fertility Insurance prediction (Schall 2000). *Plasmodium mexicanum* is peculiar, however, in that infections appear to never be cleared (Bromwich and Schall 1986), indicating a weak or non-existent host immune response, and that an unusually high number of gametocytes are common in the blood (Bromwich and Schall 1986, Taylor and Read 1997), which may prevent “low” gametocytemia from reducing fertilization rates for this species.

Overview of Dissertation

The study of malaria parasite sex ratios will broaden the taxonomic representation of empirical tests of sex ratio theory and has the potential to help predict the degree of multi-clonality and reduce parasite prevalence. Much progress has already been made in this area, especially relating to fertility insurance, but many questions remain unanswered, such as what the source of among-species variation in sex ratios is, how

common it is that species facultatively alter their sex ratio in response to co-infecting clones, and why some systems show a relationship between prevalence and sex ratio and others don't. More detailed data from additional species should help us begin to address some of these questions.

For that reason, the work presented in this dissertation focuses on testing the predictions and some assumptions of LMC and fertility insurance for a malaria parasite. Specifically, I use the lizard malaria parasite *P. mexicanum*, which has a number of traits that make it particularly useful for furthering our understanding of sex ratio evolution in malaria parasites. First, there is documented variation in both sex ratios (Osgood and Schall 2004) and clonal diversity (Vardo and Schall 2007) among infections for this species. While previous research showed no relationship between predicted clonal diversity and sex ratio (Osgood and Schall 2004), new molecular markers have been developed since that time (Schall and Vardo 2007) that allow direct estimation of clonal diversity and more detailed tests of LMC. Second, the parasite and its hosts the western fence lizard *Sceloporus occidentalis* and sand flies in the genus *Lutzomyia* have been studied by Schall and his students at the same site for over 30 years (Vardo and Schall 2007), providing a wealth of information on the basic biology of the parasite and its hosts. This long-standing research program has included almost yearly sampling, yielding an abundance of samples that can be exploited to address longer-term questions. Third, it is a natural host-parasite association with established methods for experimental manipulation and genetic testing. In this regard, research on this parasite can provide a useful complement to studies on lab-maintained clones such as the rodent malaria parasites *P. chabaudi*, *P. berghei*, and *P. vinckei*, which have undergone manipulation to

isolate and store clones that are viable in a lab setting. Additionally, these clones are studied in laboratory mice rather than their native host, the thick-knee rat. Research on *P. mexicanum* also has advantages relative to studies of the human malaria parasites like *P. falciparum*, which, while often studied in natural infections, are subject to artificial selection from antimalarial drugs (which appear to alter sex ratios, Sowumni et al. 2009a) and are not generally amenable to experimental manipulation due to the involvement of human subjects. Other natural systems, such as *Haemoproteus* and *Leucocytozoon*, often lack the availability of genetic markers (useful for estimating clone numbers per infection) or established methods for experimentation.

The central goal of this dissertation is to determine whether LMC can account for the variation in sex ratios observed for *P. mexicanum*. Selection could produce adaptive sex ratios in one of two ways: malaria parasites may be able to detect the number and relative proportions of other clones in the same infection and adjust their sex ratios accordingly, thus displaying phenotypic plasticity. Alternatively, they may have sex ratios that are locally adapted to the average degree of selfing in a population (Figure 3, Schall 2009). Chapters 2 – 4 of this dissertation test the predictions of LMC assuming phenotypic plasticity. The first prediction tested is that the sex ratio of single clone infections is female-biased (Chapter 2) and depends only on male fecundity (Chapter 3). I then address whether infections with multiple clones produce a higher sex ratio using both natural and experimental infections (Chapter 4). While the LMC model makes specific, quantitative sex ratio predictions, limitations in our ability to assess the absolute probability of selfing lead me to test these predictions qualitatively rather than quantitatively. This is certainly a limitation relative to the precision of the tests done in

laboratory systems (e.g. *P. chabaudi*, Reece et al. 2008), but the test should still provide valuable insight into whether the parasite is shifting its sex ratio in response to clonal diversity in a natural system. If no sex ratio adjustment is observed among infections, it is possible that sex ratios are locally adapted. I test for this possibility in Chapter 5 by comparing the sex ratios of populations that are far enough apart for genetic differentiation to be likely and that have sustained differences (over 30+ years) in the proportion of hosts infected. In this chapter I also test the assumption that differences in parasite prevalence lead to differences in the average number of clones per infection, an assumption that has been made previously in this type of study without validation (Read et al. 1995, Shutler et al. 1995).

If sex ratios are not fully explained by LMC, it is possible that sex ratios are either constrained so that they cannot produce an adaptive sex ratio or that malaria parasites do not fit the assumptions of LMC. Fertility insurance addresses a specific case of broken assumptions, and is included as a separate possibility in Fig. 3 because extensions to LMC have already been developed and tested for malaria parasites relating to this specific group of broken assumptions. Fertility insurance predicts an increased investment in males (relative to LMC) in response to factors reducing the probability of there being enough males to mate with all females. Much of the data supporting fertility insurance in other malaria species has related low gametocyte density to sex ratio, which I also tested for in a number of studies (Chapters 2 – 5). I discuss a number of other factors that might be important for Fertility Insurance in Chapter 4.

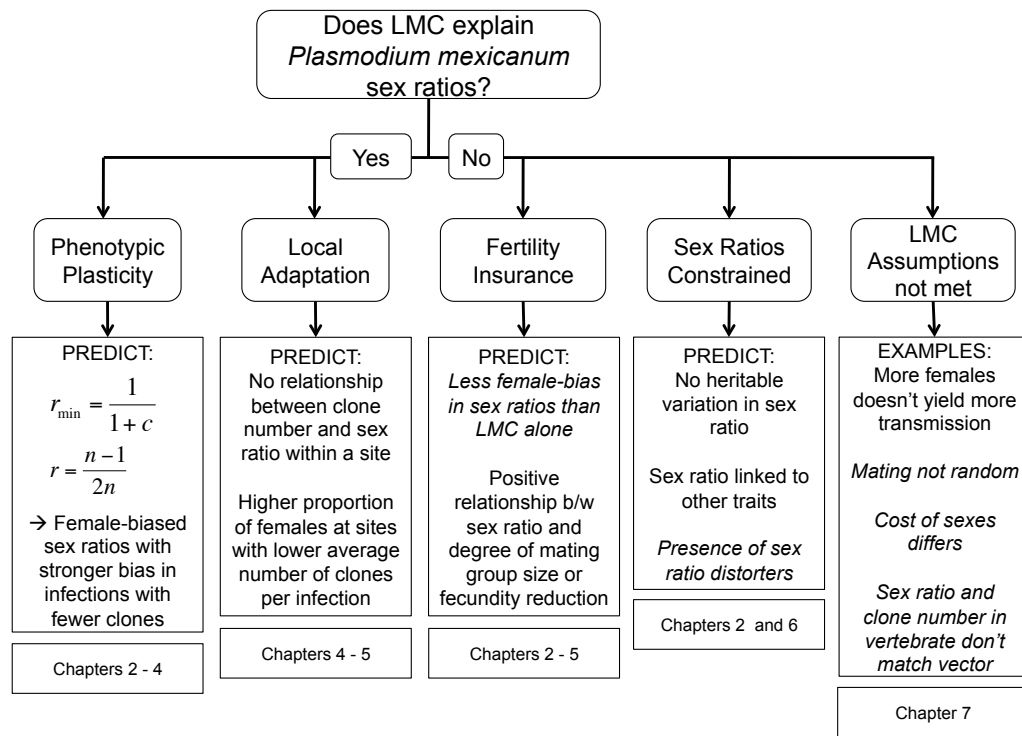


Figure 3: Overview of research question, alternative hypotheses and predictions, and chapters in this dissertation in which these topics are discussed.

Sex ratios may be constrained by the system of sex determination, a lack of heritable variation in sex ratios, the presence of sex ratio distorters, or developmental links with other traits. The mechanism of sex determination in malaria parasites is not currently known, but is not genetic (Baker 2010, Liu et al. 2011), and I know of no reference to sex ratio distorters in malaria parasites. In Chapter 6, I describe a study focusing on the relationship between sex ratios and other life history traits, which could reveal developmental links if they exist. This chapter also provides evidence for heritable variation in the sex ratio, as does Chapter 2.

Finally, there are a number of other assumptions of LMC that may be broken and could contribute to deviations from the predictions of that model. Read et al. (1992) summarize many of these assumptions in the context of malaria parasites, and a few examples are listed in Fig. 3. One assumption that has not received as much attention as it perhaps merits is the assumption that female-biased sex ratios increase genetic representation in future generations. This assumption is fundamental and implies that patches with more female-biased sex ratios overall produce more mated females that successfully produce offspring of their own. In Chapter 7, I take a closer look at this assumption. I make a case for why it might not apply well to malaria parasites, and develop a mathematical model to determine how deviations from this assumption might affect sex ratio evolution.

References

- Anderson, R. M. and R. M. May. 1991. *Infectious Diseases of Humans: Dynamics and Control*. Oxford University Press, New York.
- Anderson, T. J., R. E. Paul, C. A. Donnelly and K. P. Day. 2000. Do malaria parasites mate non-randomly in the mosquito midgut? *Genetical Research* 75: 285-296.
- Baker, D. A. 2010. Malaria gametocytogenesis. *Molecular and Biochemical Parasitology* 172: 57-65.
- Boudin, C., M. Olivier, J. F. Molez, J. P. Chiron and P. Ambroise-Thomas. 1993. High human malarial infectivity to laboratory bred *Anopheles gambiae* in a village in Burkina Faso. *American Journal of Tropical Medicine and Hygiene* 48: 700-706.
- Bromwich, C. R. and J. J. Schall. 1986. Infection dynamics of *Plasmodium mexicanum*, a malaria parasite of lizards. *Ecology* 67: 1227-1235.
- Bull, J. J. and E. L. Charnov. 1988. How fundamental are Fisherian sex ratios? *Oxford Surveys in Evolutionary Biology* 5: 96-135.
- Bulmer, M. G. and P. D. Taylor. 1980. Sex ratio under the haystack model. *Journal of Theoretical Biology* 86: 83-89.

Burkot, T. R., J. L. Williams and I. Schneider. 1984. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown in vitro from the same isolate. Transactions of the Royal Society of Tropical Medicine and Hygiene 78: 339-341.

Carter R. and L. H. Miller. 1979. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. Bulletin of the World Health Organization 57 S1: 37-52.

Charnov, E. L. The Theory of Sex Allocation. Princeton University Press, Princeton. Monographs in Population Biology 18.

Clark, A. B. 1978. Sex ratio and local resource competition in a prosimian primate. Science 201: 163-165.

Colwell, R. K. 1981. Group selection is implicated in the evolution of female-biased sex ratios. Nature 290: 401-404.

Dye, C. and H. C. F. Godfray. 1993. On sex ratio and inbreeding in malaria parasite populations. Journal of Theoretical Biology 161: 131-134.

Edwards, A. W. F. 1998. Natural selection and the sex ratio: Fisher's sources. American Naturalist 151: 564-569.

Edwards, A. W. F. 2000. Carl Düsing (1884) on the regulation of the sex-ratio. Theoretical Population Biology 58: 255-257.

Fisher, R. A. 1930. The Genetical Theory of Natural Selection. Dover Publications, New York.

Gardner, A., S. Reece. S. West. 2003. Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. Journal of Theoretical Biology 223: 515-521.

Hartl, D. L. 1971. Some aspects of natural selection in arrhenotokous populations. American Zoologist 11: 309-325.

Hamilton, W. D. 1967. Extraordinary sex ratios. Science 156: 477-488.

Herbers, J. M. 1984. Queen-worker conflict and eusocial evolution in a polygynous ant species. Evolution 38: 631-643.

James, S. P. 1931. Some general results of a study of induced malaria in England. Transactions of the Royal Society of Tropical Medicine and Hygiene 24: 477-538.

- Liu, Z., J. Miao and L. Cui. 2011. Gametocytogenesis in malaria parasite: commitment, development and regulation. *Future Microbiology* 6: 1351-1369.
- Martinsen, E. S., S. L. Perkins, J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* 47: 261-273.
- Maynard Smith, J. 1982. *Evolution and the Theory of Games*. Cambridge University Press, New York.
- Merino, S., G. Tomás, J. Moreno, J. J. Sanz, E. Arriero and C. Folgueira. 2004. Changes in *Haemoproteus* sex ratios: fertility insurance or differential sex lifespan? *Proceedings of the Royal Society of London Series B: Biology* 271: 1605-1609.
- Mitri, C., I. Thiery, C. Bourgouin and R. E. L. Paul. 2009. Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates. *Proceedings of the Royal Society of London Series B: Biology* 276: 3721-3726.
- Orzack, S. H. 2002. Using sex ratios: the past and the future. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 383-398). Cambridge University Press, New York.
- Osgood, S. M., R. J. Eisen and J. J. Schall. 2002. Gametocyte sex ratio of a malaria parasite: experimental test of heritability. *Journal of Parasitology* 88: 494-498.
- Osgood, S. M., R. J. Eisen, A. R. Wargo and J. J. Schall. 2003. Manipulation of the vertebrate host's testosterone does not affect gametocyte sex ratio of a malaria parasite. *Journal of Parasitology* 89: 190-192.
- Osgood, S. M. and J. J. Schall. 2004. Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* 128: 23-29.
- Paperna, I. and I. Landau. 1991. *Haemoproteus* (Haemosporidia) of lizards. *Bulletin du Museum National d'Histoire Naturelle Section A, Zoologie, Biologie et Ecologie Animales* 13: 309-349.
- Paul, R. E. 2000. Sex determination in malaria parasites. *Science* 287: 128-131.
- Paul, R. E. L., P. T. Brey and V. Robert. 2002. *Plasmodium* sex determination and transmission to mosquitoes. *Trends in Parasitology* 18: 32-38.
- Paul, R. E. and K. P. Day. 1998. Mating patterns of *Plasmodium falciparum*. *Parasitology Today* 14: 197-202.

- Paul, R. E., M. J. Packer, M. Walmsley, M. Lagog, L. C. Ranford-Cartwright, R. Paru and K. P. Day. 1995. Mating patterns in malaria populations of Papua New Guinea. *Science* 269: 1709-1711.
- Paul, R. E. L., A. Raibaud and P. T. Brey. 1999. Sex ratio adjustment in *Plasmodium gallinaceum*. *Parassitologia* 41: 153-158.
- Pen, I. and F. J. Weissing. 2002. Optimal sex allocation: steps towards a mechanistic theory. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 26-45). Cambridge University Press, New York.
- Pickering, J., A. F. Read, S. Guerrero and S. A. West. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research* 2: 171-184.
- Read, A. F., M. Anwar, D. Shutler and S. Nee. 1995. Sex allocation and population structure in malaria and related parasitic protozoa. *Proceedings of the Royal Society of London Series B: Biology* 260: 359-363.
- Read, A. F. and K. P. Day. 1992. The genetic structure of malaria parasite populations. *Parasitology Today* 8: 239-242.
- Read, A. F., A. Narara, S. Nee, A. K. Keymer, and K. P. Day. 1992. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Read, A. F., T. G. Smith, S. Nee and S. A. West. 2002. Sex ratios of malaria parasites and related protozoa. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 314-332). Cambridge University Press, New York.
- Reece, S. E., D. R. Drew and A. Gardner. 2009. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614.
- Reece, S. E., A. B. Duncan, S. A. West and A. F. Read. 2003. Sex ratios in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* 127: 519-525.
- Robert, V., A. F. Read, J. Essong, T. Tchuinkam, B. Mulder, J.-P. Verhave and P. Carnevale. 1996. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 621-624.
- Robert, V., C. S. Sokhna, C. Rogier, F. Arieu, and J.-F. Trape. 2003. Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* 127: 1-8.
- Schall, J. J. 1989. The sex ratio of *Plasmodium* gametocytes. *Parasitology* 98: 343-350.

- Schall, J. J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.
- Schall, J. J. 2009. Do malaria parasites follow the algebra of sex ratio theory? *Trends in Parasitology* 25: 120-123.
- Schall, J. J. and A. M. Vardo. 2007. Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Molecular Ecology Notes* 7: 227-229.
- Shutler, D., G. F. Bennett and A. Mullie. 1995. Sex proportions of *Haemoproteus* blood parasites and local mate competition. *Proceedings of the National Academy of Sciences* 92: 6748-6752.
- Shutler, D. and A. F. Read. 1998. Local mate competition, and extraordinary and ordinary blood parasite sex ratios. *Oikos* 82: 417-424.
- Seger, J. and J. W. Stubblefield. 2002. Models of sex ratio evolution. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 2-25). Cambridge University Press, New York.
- Smalley, M. E. and R. E. Sinden. 1977. *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* 74: 1-8.
- Sober, E. 2001. Sex ratio theory, ancient and modern - an 18 century debate about intelligent design and the development of models in evolutionary biology. In: Riskin, J. (Ed.) *Genesis Redux: Essays in the History and Philosophy of Artificial Life* (pp. 131-162) The University of Chicago Press, Chicago.
- Sowunmi, A., S. T. Balogun, G. O. Gbotosho and C. T. Happi. 2009a. *Plasmodium falciparum* gametocyte sex ratios in symptomatic children treated with antimalarial drugs. *Acta tropica* 109: 108-117.
- Sowunmi, A., G. O. Gbotosho, C. T. Happi, O. A. Folarin, and S. T. Balogun. 2009b. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitology International* 58: 438-443.
- Stubblefield, J. W. and J. Seger. 1990. Local mate competition with variable fecundity: dependence of offspring sex ratios on information utilization and mode of male production. *Behavioral Ecology* 1: 68-80.
- Taylor, L. H. and A. F. Read. 1997. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitology Today* 13: 135-140.

Taylor, P. D. 1981. Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature* 291: 64-66.

Taylor, P. D. and M. Bulmer. 1980. Local mate competition and the sex ratio. *Journal of Theoretical Biology* 86: 409-419.

Trager, W., M. Tershakovec, L. Lyandvert, H. Stanley, N. Lanners and E. Gubert. 1981. Clones of the malaria parasite *Plasmodium falciparum* obtained by microscopic selection: their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes. *Proceedings of the National Academy of Science* 78: 6527-6530.

Trivers, R. L. 1974. Parent-offspring conflict. *American Zoologist* 14: 249-264.

Trivers, R. L. and D. E. Willard. 1973. Natural selection of parental ability to vary the sex ratio of offspring. *Science* 179: 90-92.

Vardo, A. M. and J. J. Schall. 2007. Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Molecular Ecology* 16: 2712-2720.

West, S. 2009. Sex allocation. Princeton University Press, Princeton.

West, S. A. and E. A. Herre. 2002. Using sex ratios: why bother? In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 399-413). Cambridge University Press, New York.

West, S. A., S. E. Reece and A. F. Read. 2001. Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology* 17: 525-531.

West, S. A., T. G. Smith, S. Nee and A. F. Read. 2002. Fertility insurance and the sex ratios of malaria and related Hemosporidion blood parasites. *Journal of Parasitology* 88: 258-263.

Williams, G. C. 1966. *Adaptation and Natural Selection*. Princeton University Press, Princeton.

Wilson, D. S. and R. K. Colwell. 1981. Evolution of sex ratios in structured demes. *Evolution* 35: 882-897.

Yamaguchi, Y. 1985. Sex ratios of an aphid subject to local mate competition with variable maternal condition. *Nature* 318: 460-462.

Zollner, G. E., N. Ponsa, G. W. Garman, S. Poudel, J. A. Bell, J. Sattabongkot, R. E. Coleman and J. A. Vaughan. 2006. Population dynamics of sporogony for *Plasmodium*

vivax parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. Malaria Journal 5: 68.

CHAPTER 2: Gametocyte sex ratio in single-clone infections of the malaria parasite

Plasmodium mexicanum

Allison T. Neal and Joseph J. Schall

Summary

Sex ratio theory predicts that malaria parasites should bias gametocyte production toward female cells in single-clone infections because they will experience complete inbreeding of parasite gametes within the vector. A higher proportion of male gametocytes is favoured under conditions that reduce success of male gametes at reaching females such as low gametocyte density or attack of the immune system later in the infection. Recent experimental studies reveal genetic variation for gametocyte sex ratio in single-clone infections. We examined these issues with a study of experimental single-clone infections for the lizard malaria parasite *Plasmodium mexicanum* in its natural host. Gametocyte sex ratios of replicate single-clone infections were determined over a period of 3-4 months. Sex ratios were generally female biased, but not as strongly as expected under simple sex ratio theory. Gametocyte density was not related to sex ratio, and male gametocytes did not become more common later in infections. The apparent surplus of male gametocytes could be explained if male fecundity is low in this parasite, or if rapid clotting of the lizard blood reduces male gamete mobility. There was also a significant clone effect on sex ratio, suggesting genetic variation for some life history trait, possibly male fecundity.

Introduction

Sex ratio theory, a productive focus in evolutionary biology, seeks to understand how natural selection leads to an optimal allocation of reproductive effort to male and female offspring among individuals of a dioecious species and what ecological and

evolutionary factors lead to variation in the population sex ratio (Charnov, 1982).

Although the theory was originally developed for large multicellular organisms such as plants and animals, one influential model, Local Mate Competition (LMC) (Hamilton, 1967), applies well to the reproductive system of malaria parasites (*Plasmodium* and related genera). This model posits that any local population with limited genetic variation and high levels of inbreeding should produce a female-biased sex ratio that will reduce competition among brothers for matings with sisters. For a strictly clonal population in which all individuals are genetically identical, the optimal sex ratio would be just enough males to mate with all females, and the sex ratio would therefore be related to the number of mates possible for a single male. In contrast, genetically diverse populations, which experience low levels of inbreeding, should produce more equal proportions of males and females (West *et al.* 2001; Read *et al.* 1992).

Malaria parasites undergo multiple cycles of asexual replication within the vertebrate host, eventually producing male or female sex cells, the microgametocytes and macrogametocytes. Within the vector's midgut, each female gametocyte develops into a single female gamete and each male gametocyte produces one to several flagellated male gametes. Male fecundity is therefore the number of successful male gametes produced by a single microgametocyte. The male and female gametes quickly combine within the midgut of the vector, completing the sexual stage of malaria's life cycle (Garnham, 1966). The gametes competing for fertilization are limited to those taken up by a single vector, and therefore the breeding population of a malaria parasite is partitioned among individual insect vectors where LMC will occur (West *et al.* 2001). The algebra of sex ratio theory predicts the expected gametocyte sex ratio in the vertebrate hosts based on

the genetic (clonal) diversity present and likely degree of inbreeding. For example, when a single clone of parasites is present, just enough male gametocytes should be produced to mate with all the female gametocytes within the vector, and this value would depend on the fecundity of male gametocytes (Read *et al.* 1992; Reece *et al.* 2008; Schall, 2009).

West *et al.* (2001) and Gardner *et al.* (2003) noted that environmental conditions that reduce the probability of male gametes finding and combining with female gametes would favour “fertility insurance” with an increase in investment in male gametocytes. For example, as the vertebrate host mounts an immune response to an infection, immune factors from the vertebrate carry over into the blood meal and attack the parasite’s male gametes (the “transmission blocking immunity” of Carter *et al.* 1979). This effect should be more pronounced late in the course of an infection when the host immune system detects the presence of the parasite. Low density of gametocytes in the vertebrate blood, a common situation for many malaria infections (Taylor and Read, 1997), would also lead to an apparent “surplus” of male gametocytes relative to that expected by the simple LMC model because of the difficulty faced by the very few male gametes in finding a female gamete during the few minutes available for mating in the vector.

In summary, the LMC model with fertility insurance predicts that single-clone infections of a malaria parasite will produce a female-biased sex ratio (with the precise bias depending on the fecundity of the males). This bias should be reduced or absent when gametocyte density is low, and will fade over the course of an infection due to immune system attack and resulting transmission blocking immunity. Elegant experimental studies of rodent (Reece *et al.* 2008) and avian (Paul *et al.* 1999, 2000) malaria parasites support LMC with fertility insurance. Infections of *P. chabaudi*

established in laboratory mice produce a sex ratio early in an infection that matches the algebraic predictions of the theory depending on number of clones and their relative proportions in the mouse blood. For both *P. chabaudi* and *P. gallinaceum* in domestic chickens, single-clone infections are strongly female-biased early in the infection, but switch to greater production of male gametocytes over time, perhaps because the host mounts an immune attack that could reduce the fecundity of male gametocytes. The rodent malaria experiments, however, present another intriguing result. The well-characterized laboratory clones of *P. chabaudi* tend to produce female-biased sex ratios early in an infection, as predicted by the theory, but there is consistent variation among clones in precise sex ratio. The only other suggestion of variation in gametocyte sex ratio among clones is from Burkot (1984) who found variation in a few laboratory clones of *P. falciparum*, although these were clones maintained in vitro. Variation among clones for gametocyte sex ratio could be a result of genetic variation for some factor that drives sex ratio among clones, perhaps male gametocyte fecundity, gametocyte density, or effect on erythrocyte density (Reece *et al.* 2008).

We investigated the gametocyte sex ratio of a natural parasite-host association, *P. mexicanum* in its reptile host, the western fence lizard, *Sceloporus occidentalis*. We studied replicate experimental single-clone infections of ten genetic clones to test if (a) infections with only a single clone produce female-biased gametocyte sex ratios; (b) the gametocyte sex ratios of infections change over time to produce more males; (c) gametocyte density is correlated with sex ratio; (d) there is clonal (genetic) variation for sex ratio. This is the first such study of single-clone infections of a natural parasite-host system.

Materials and Methods

Experimental infections

Blood dots and smears for this study were obtained from two experiments, both conducted at the University of California Hopland Research and Extension Center near Hopland California in southern Mendocino County, California, USA. Detailed descriptions of the experimental design of these experiments can be found in Osgood and Schall (2004) (henceforth referred to as Study I) and Vardo-Zalik and Schall (2008, 2009) (henceforth referred to as Study II). Briefly, blood from western fence lizards (*Sceloporus occidentalis*) naturally infected with *P. mexicanum* was used to initiate replicate infections in previously noninfected recipient lizards. Blood from a donor lizard with a standard number of asexual stage parasites mixed with saline was injected into the peritoneal cavity of recipient lizards. This method of initiating infections is described in detail in Eisen and Schall (2000). Experimental lizards were kept outdoors in vector-proof cages and blood was taken from a toe clip every 7 (Study I) or 10 (Study II) days for 4 (Study I) or 3 (Study II) months during the summer. Blood dots were dried and frozen and fresh blood was used to prepare a thin smear, which was treated with Giemsa stain (Schall, 1989).

For our study we used only the infections initiated from donors that were judged to contain a single parasite genotype. These single-clone infections were identified by analyzing 5 (Study I) or 3 (Study II) microsatellite markers using PCR primers and conditions presented by Schall and Vardo (2007). Study I had 7 single-clone donors, which were each used to initiate 3, 4, or 5 recipient infections, for a total of 31 induced infections. Study II had 3 single-clone donors, which were used to initiate 3 or 7

recipient infections each, for a total of 17 induced infections. Examination of the microsatellite allele data showed all clones were distinct for the two studies. Large and equal sample sizes were not possible due to the small size of donor lizards and the relative scarcity of single-clone infections at the study site (Vardo and Schall, 2009).

Gametocyte sex ratio

We determined gametocyte sex ratio by scanning blood smears at 1000x and scoring each gametocyte as male or female, taking care to sample gametocytes from all areas of the smear. Each slide was examined until 100 mature gametocytes were scored or a maximum of 1 hour. Male and female gametocytes can be readily differentiated based on their appearance after staining as noted by Schall (1989). To assure accurate scoring of gametocytes, all slides were counted by one investigator (ATN), and two other investigators scored samples of the smears; all three investigators produced very similar results.

Time and gametocytaemia effect on sex ratio

Gametocyte sex ratio and gametocytaemia (gametocytes per 1000 erythrocytes examined under 1000x) were determined for every recipient infection in Study I 5, 6, 10 and 11 weeks after each lizard was inoculated with infected blood to investigate possible time and gametocytaemia effects on sex ratio. In addition, one recipient infection per donor from Study I was selected and the sex ratio was determined for every available slide to determine if sex ratio varied over the course of an infection.

Genetic variation for sex ratio

We used gametocyte sex ratio counts from Study I 5, 6, 10 and 11 weeks post inoculation (above) as well as 3 and 9 weeks after gametocytes became patent in the blood to determine if there was genetic variation for sex ratio. Gametocyte patency was based on a one minute scan at 1000x. Gametocytes first became patent in the blood 1 to 7 weeks post inoculation, with the majority of infections (18 out of 31) becoming patent 3 to 4 weeks post inoculation. We used sex ratio counts from a series of weeks after gametocyte patency in addition to a series of weeks post inoculation because we noted that infections differed in the time it took for gametocytes to become patent in the blood of the host and concluded this difference in establishment time might affect the sex ratio of the infection.

Multiple dates were sampled in case confounding factors early in an infection (such as a long establishment time) or late in an infection (such as interactions with the lizard immune system) might mask a clone effect. We also used sex ratio counts from Study II 11 weeks post inoculation and 3 weeks post gametocyte patency (fewer blood smears were made in Study II, so fewer dates were possible) to determine if the presence or absence of a genetic effect on sex ratio was also observed in a different set of clones.

Statistical analysis

Statistical analysis was performed using JMP 8 or SAS 9.2 (SAS Institute, Cary, NC, USA). To test for any role of age of the infection, gametocytaemia, and clone on gametocyte sex ratio, data for Study I (for which a longer series of sample dates was available) 5, 6, 10 and 11 weeks post inoculation were analyzed using a Generalized

Estimating Equation (GEE) fitted to a binomial error and logit-link function for gametocyte sex ratio (as the dependent variable) (SAS). GEE take into account repeated measurements of the same infection over a series of weeks. Entered into the analysis for each slide examined were number of male and total number of gametocytes counted, sample time, gametocytaemia (gametocytes per 1000 erythrocytes), and clone. To further examine a possible genetic influence on gametocyte sex ratio, all clones (Studies I and II separately) were analyzed using a Generalized Linear Model (GLM) at each date, again with sex ratio modeled with binomial errors and logit-link function (JMP). For seven infections with gametocyte sex ratio for each sample period, we plotted 90% confidence intervals for proportion data for each sample.

Results

Overall gametocyte sex ratio

A total of 48 experimentally induced infections were followed from Study I and II, with 297 blood samples examined. Some lizards died before the entire sequence of smears was taken over time, and some smears did not contain sufficient gametocytes to calculate sex ratio. Of the 297 smears examined, 287 allowed sex ratio counts, with a sample size of at least 100 for 245 smears, 75-99 for 4, 50-74 for 20, and < 50 for 18 (these had low parasite densities). Gametocyte sex ratios had a mean/median/mode proportion male of 0.44/0.44/0.42 and infections in which at least 100 mature gametocytes were counted ranged from a proportion male gametocytes of 0.28 to 0.63 (Fig. 4).

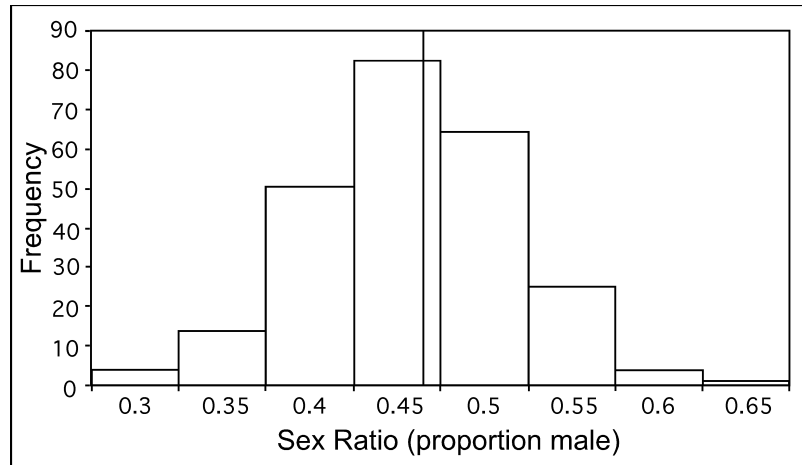


Figure 4: Sex ratios of single-clone replicate infections of the malaria parasite *Plasmodium mexicanum* in its natural host, the western fence lizard *Sceloporus occidentalis*. Graph includes sex ratios of all experimentally induced single-clone infections from Studies I and II in which at least 100 mature gametocytes were scored as male or female. Data are from 48 infections over time for a total of 245 gametocyte sex ratios scored. A reference line is included at the mean/median value (.44).

Table 2: Summary of seven infections of different clones of the malaria parasite *Plasmodium mexicanum* in its natural lizard host followed over time. Start and end times indicate the number of weeks after inoculation with infected blood or after gametocyte patency we started and ended recording sex ratio. Sex ratio counts were performed once a week during the specified period, and the total number of slides for which counts were performed is noted under “Total Weeks”. The asterisks indicate that one slide was missing from the series.

Clone	Inoculation		Patency		Total Weeks
	Start	End	Start	End	
1	7	19	2	14	13
2	5	19	1	15	15
3	5	18	1	15	13*
4	5	18	3	16	14
5	5	17	1	13	13
6	5	17	0	12	12*
7	5	14	1	10	10

Gametocyte sex ratio over time and effect of gametocytemia

Overall, gametocyte sex ratio was not related to sample time (GEE, $P = 0.144$).

Because of low gametocyte numbers early in some infections and the death of a few lizards, the number of samples available for counting gametocytes was reduced at

different time periods (post inoculation and post patency). Sample sizes are given in Table 3.

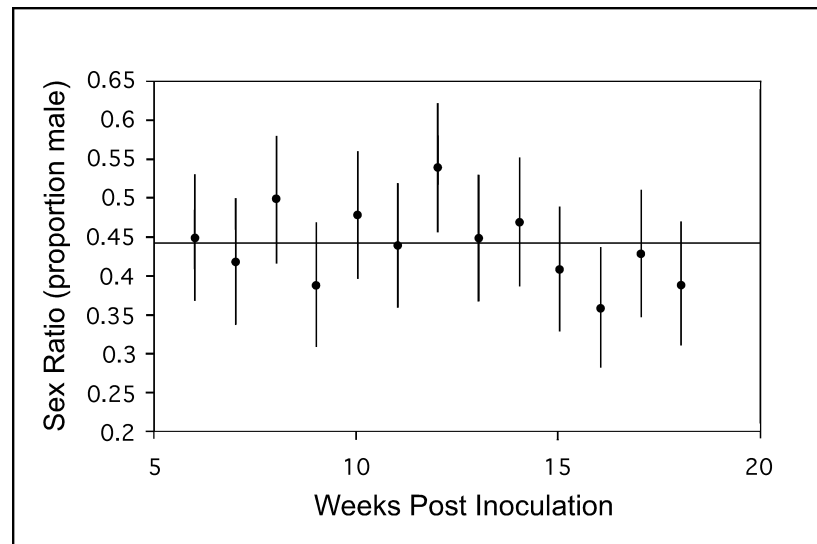


Figure 5: Gametocyte sex ratio of the malaria parasite *Plasmodium mexicanum* in an experimentally infected lizard (*Sceloporus occidentalis*) six to eighteen weeks after initiation of infection via injection of infected blood from a donor lizard. Sex ratio counts are from an individual lizard from Study I infected with a single clone of a parasite. Six additional infections of 6 different clones from Study I were also sampled and showed a similar lack of trend in sex ratio over time. Each point represents a single count and error bars represent a 90% confidence interval (calculated for proportion data) and a reference line showing the grand mean for all samples is shown.

Gametocyte sex ratio of 7 infections was followed for ~13 weeks (~5 weeks to 18 weeks post inoculation, 1 week to 14 weeks post gametocyte patency). Table 2 shows the start and end times for each infection. Each of these 7 infections was initiated with blood taken from different donor lizards (and thus different parasite genotypes) from Study I, and thus represented different genetic clones in each infection. The start and end dates of our counts are not constant because time of first patency differed somewhat among infections and infection initiation dates varied (altering the end dates possible). Fig. 5 shows a sample infection of the seven with 90% confidence intervals for the calculated proportion of male gametocytes. The result drawn from this figure is consistent across all 7 infections: confidence intervals overlapped broadly. Note that if

there were errors in counting immature gametocytes (that appear more female in morphology [Schall, 1989]), then sex ratio should increase in proportion of males over time; this was not observed.

Median gametocytaemia was 4.5 gametocytes per 1000 erythrocytes and ranged from 0 to 117 gametocytes per 1000 erythrocytes. Sex ratio was not related to gametocytaemia (114 samples for the 31 experimental infections of Study I; GEE $P = 0.402$). Sample sizes for each date are in Table 3.

Table 3: Summary of the presence or absence of a clone effect on the sex ratio of single-clone infections of the malaria parasite *Plasmodium mexicanum* in its natural host, the western fence lizard *Sceloporus occidentalis*. Infections were taken from 2 previous studies. All infections were experimentally initiated and followed over the course of 3 to 4 months. Sex ratios for each infection were determined a standard number of weeks post inoculation or post gametocyte patency (based on a one minute scan). Multiple times were sampled because factors early in the infection (such as a long establishment time) or late in the infection (such as the parasite's response to attacks from the immune system) could mask any a genotype effect. P values are based on GLM.

Study	Time	N	Clone effect?	P
I	5 weeks post inoculation	28	no	0.0536
I	5 weeks post inoculation	30	no	0.2725
I	10 weeks post inoculation	27	no	0.3426
I	11 weeks post inoculation	26	yes	0.0002
II	11 weeks post inoculation	15	yes	0.0179
I	3 weeks post gametocyte patency	31	no	0.4083
II	3 weeks post gametocyte patency	16	yes	0.0233
I	9 weeks post gametocyte patency	21	no	0.1139

Genetic variation in gametocyte sex ratio

When clone was entered into the GEE analysis with time and gametocytaemia for Study I, there is no significant effect ($P = 0.058$). However, the P value suggested that a Type II error was possible by combining all time periods. Separating time periods for both studies, a GLM showed a significant clone effect in Study II for gametocyte sex ratio at both times at which counts were performed. Study I showed a significant clone effect on gametocyte sex ratio at the latest date sampled (11 weeks post inoculation), but

not at any of the other dates (5, 6, and 10 weeks post inoculation, 3 and 9 weeks post patency). These findings are shown in Table 3 and Fig. 6.

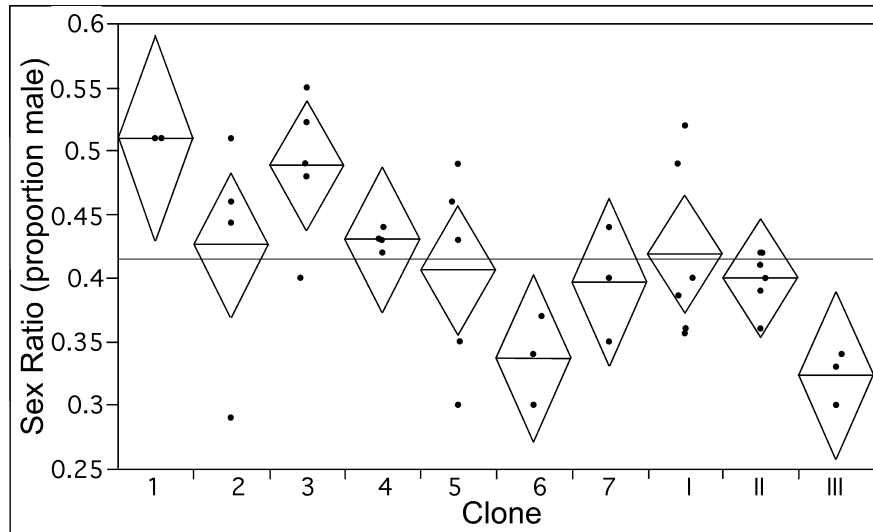


Figure 6: Clone effect on gametocyte sex ratio of the malaria parasite *Plasmodium mexicanum* in experimental single clone infections 11 weeks after initiation of the infection. Clones 1-7 are from Study I. Clones I-III are from Study II. Points represent individual infections and are slightly shifted horizontally to make all points visible. Diamonds show mean line at the center and 95% confidence interval vertically between tips. The grand mean is displayed as a reference line.

Discussion

Predictions of sex ratio theory

Within sex ratio theory, the model of LMC predicts that a single clone of malaria parasites replicating within a vertebrate host will produce a female-biased gametocyte sex ratio. However, several factors would drive gametocyte sex ratio toward greater production of male cells. First, male fecundity, or the number of gametes produced by a male gametocyte, could be low, and thus even the process of LMC would result in no female bias. Second, if the mobility or viability of male gametes is reduced by any environmental factor in the blood meal taken by the vector, an apparent “surplus” of males will be produced, even in single-clone infections. For example, immune system

carry-over in the vector could become more severe over time, thus favouring production of more male gametocytes. Last, low density of gametocytes in the vertebrate host's blood would also result in fertility insurance and shifting of the sex ratio away from a female bias.

These predictions assume that malaria parasites use a phenotypically plastic strategy such that the parasite can monitor and respond to changing environmental conditions within the vertebrate host, including clonal diversity. This appears to be the case for *P. chabaudi* in experimental infections in laboratory mice (Reece et al. 2008). The rodent malaria parasite appears to detect both number of coexisting clones and their relative abundance in an infection, and shifts gametocyte sex ratio to match very closely the algebraic predictions of LMC (Schall, 2009). The *P. chabaudi* infections also shift gametocyte sex ratio over time toward more male gametocytes (consistent with an induced immune attack, although still not conclusively demonstrated), as well as differences in gametocyte density in the blood. The avian malaria parasite, *P. gallinaceum*, appears to monitor indirectly the activity of the bird host's immune system and shifts to a less female-biased gametocyte sex ratio when erythrocytes are being rapidly cleared and replaced (Paul et al. 1999, Paul 2000). In contrast, malaria parasites could also evolve a fixed strategy of an optimal gametocyte sex ratio based on prevailing clonal diversity and predictable changes in environmental conditions over the course of the infection. In this case, some infections may reveal what appears to be a “surplus” of male gametocytes. Selection may favour a balanced genetic polymorphism for gametocyte sex ratio if conditions within infections vary substantially and only a fixed strategy is possible. We tested these predictions by producing replicate single-clone

infections of *P. mexicanum* in its natural lizard host, the western fence lizard. This study has the advantage of being a natural parasite-host system, but also suffers additional complexity compared to the rodent or bird malaria laboratory model systems (below).

Overall sex ratio

The gametocyte sex ratios observed in this study averaged only marginally female biased. The distribution of sex ratios determined for the experimental infections with a single clone of parasite is similar to that seen for natural infections of *P. mexicanum* previously sampled (one sample for each infected lizard) at the field site (mean 0.46 males, range 0.24 – 0.72; Schall, 1996). These results predict an effective male fecundity ranging from 0.61 to 2.6 gametes, with most common male fecundity of 1.3 gametes based on the LMC model (Schall, 2009). The only estimate of male fecundity for *P. mexicanum* is presented by Osgood and Schall (2004), who report the number of gametes produced by a small sample (only 19) of male gametocytes from five natural infections, with a mode of five and range of one to six gametes. This would predict a gametocyte sex ratio of 0.17 (1 male:5 females); none of the experimental infections produced a gametocyte sex ratio this strongly female biased (Fig. 4). This result contrasts with the generally significantly female bias in gametocyte sex ratios for malaria parasites of humans (Read et al., 1992; West *et al.*, 2001), birds (Paul 2000), and rodents (Reece *et al.*, 2003), and suggests that male fecundity of *P. mexicanum* is low, fertility insurance is strongly active, or the parasite has evolved a fixed strategy that will not produce a female-biased sex ratio even when only a single clone of parasites is present.

Fertility insurance: infection over time

We noted no increase in proportion of male gametocytes over the course of the *P. mexicanum* infections. This contrasts with the pattern seen in bird or rodent malaria infections in which gametocyte sex ratio shifts from a strong female bias to more equal proportion of male and female cells over time (Paul *et al.* 1999, Paul 2000; Reece *et al.* 2008), and is particularly apparent for infections that are self curing (Paul *et al.* 1999, Paul 2000). Nothing is known concerning the response of the reptilian immune system to infection with malaria parasites (or for that matter, very little is known concerning the lizard immune response to any parasite infection). *P. mexicanum* occurs in chronic infections in fence lizards under natural conditions, with the course of infection resulting in a stable parasitaemia lasting at least several months (Bromwich and Schall 1986). The parasitaemia in these stable infections ranges over three orders of magnitude. Likewise, gametocyte sex ratio also varies among infections and remains constant for most infected lizards (Schall 1989). These findings suggest that lizards do not mount a strong immune response to the parasite, at least not one that interferes with the male gametes in the vector's bloodmeal. Osgood *et al.* (2003) found that experimental manipulation of testosterone levels in the lizards, which should have an effect on the lizard's immune system, had no effect on the sex ratio, growth rate, or peak parasitaemia of *P. mexicanum*, further suggesting that the lizard's immune system does not mount a strong attack on the parasite. A weak or absent immune response would explain why sex ratio did not increase over the course of an infection.

An alternate explanation for why sex ratio did not increase over the course of an infection for *P. mexicanum* is that this parasite's gametocytes are long-lived such that most gametocytes, even those produced very early in an infection, may eventually experience attack of immune products in the vector. Reece *et al.* (2003) found that the half-life of gametocytes of *Plasmodium chabaudi* is only fourteen hours, but Schall (1989) observed that *P. mexicanum* gametocytes increase in size over the course of three months in individual infections, which could indicate that gametocytes of *P. mexicanum* are significantly longer lived. More research into the longevity of gametocytes in this species needs to be conducted before we can fully evaluate this hypothesis.

Fertility insurance: gametocyte density

We found no relationship between gametocytaemia and sex ratio for the experimental *P. mexicanum* infections. Previous studies have found a correlation between sex ratio and gametocytaemia (Pickering *et al.* 2000, Robert *et al.* 2003, Reece *et al.* 2008), including for *P. mexicanum* (Schall, 2000). The correlation between gametocytaemia and sex ratio noted in previous studies with *P. mexicanum* (Schall, 2000) could have been a result of differences in genetic diversity of the infections (Schall, 2002) rather than of fertility insurance. In general, lizard malaria parasites tend to produce far more gametocytes in the host's blood than do those of bird or mammal malaria parasites (Schall, 1996; Taylor and Read, 1997). If this aspect of fertility insurance only becomes important below some lower threshold of absolute gametocyte number in the blood meal, *P. mexicanum* infections may seldom have gametocyte

densities below this level. That is, gametocytes will simply be common in most infected lizards, with large numbers taken up by the vector.

Experimental studies of transmission success of the parasite to the sandfly vectors (two species of *Lutzomyia*) reveal almost all sandflies become infected after feeding on an infected lizard's blood, and the midgut is often covered with oocysts (Fialho and Schall, 1995; Schall, 2000). Another possible confounding factor is that we measured the relative numbers of gametocytes per number of erythrocytes (gametocytaemia) rather than actual density of the gametocytes per volume of blood taken by a vector. Previous studies have found rather little variation in erythrocyte numbers per volume of blood in both infected and noninfected fence lizards (Schall, 1990), so we suspect gametocytaemia measured here is relevant for fertility insurance.

Fertility insurance: blood clotting

Another unexplored factor that could favour fertility insurance is the clotting of blood within the vector. For male gametes to locate female gametes, they must travel through the vector's blood meal to a female gamete within a few minutes. If lizard blood taken up by a vector rapidly clots, this would reduce the mobility of male gametes and could explain the apparent surplus of male gametocytes in single-clone infections (Alano and Billker, 2005). We know of no comparative studies of blood clotting for reptiles *vs.* mammals and birds. Anecdotally we have noted that fence lizard blood clots more rapidly than bird or mammal blood, and Vardo-Zalik and Schall (2008) report that blood from infected fence lizards clots more quickly than blood from lizards not infected with *P. mexicanum*. Even in heparin-treated capillary tubes, the blood often formed complete

clots within seconds, making measurement of haemoglobin difficult. This would be a result strictly of the density of the clotting blood, rather than the known killing of malaria parasite cells by platelets (Pleass, 2009) because reptiles do not produce platelets in their blood. Clearly, measurement of gamete mobility within vector blood meals to compare different species of malaria parasites would be a technical challenge.

Genetic variation in sex ratio

The experimental *P. mexicanum* infections provide evidence of genetic variation in sex ratio for a malaria parasite in its natural host from two independent studies. The clones selected for Study II appear to be more differentiated with respect to sex ratio than those in Study I since a clone effect was detectable at both dates examined in Study II. Study I showed no genetic variation for sex ratio when the four dates were analyzed together (possibly a type II error, see results), but when each date was analyzed separately, a significant clone effect on the latest date (11 weeks post inoculation) emerged. Reece *et al.* (2008) found evidence that rodent malaria parasites are able to recognize related from non-related conspecifics and adjust their sex allocation strategies accordingly. If this is also the case for *P. mexicanum* and if recognition cues are density dependent, it is possible that these cues are weak early in the infection, making it difficult for the parasite to detect the clonal diversity of the infection and respond accordingly. Such a situation could lead to a clone effect being hidden early in an infection and emerging only when the parasite is able to detect that it is alone.

Previously, such variation was found for *P. chabaudi* infections in laboratory mice (Reece *et al.* 2008) and for a few cloned lines of *P. falciparum* (Burkot, 1984). This

variation could be due to variation in male gametocyte fecundity among clones. If so, why should there be a genetic polymorphism for such an important life history trait? What would be the selective advantage for any cell to produce fewer gametes? Gametocytes that produce more gametes are able to fertilize more females, but male gametocytes that produce fewer might produce gametes more quickly or of higher quality, making them more successful in finding female gametes (Alano and Billker, 2005). Additional research into the fecundity of different genotypes is necessary to determine if variable fecundity is a probable source of variation in sex ratio between genotypes.

Phenotypic plasticity or fixed strategy?

Malaria parasites could achieve an adaptive sex ratio either by having the ability to alter gametocyte sex ratio based on conditions within each infection (such as clonal diversity or host immune attack) or by having a fixed sex ratio that is adaptive for the most common conditions within infections. There could also be a polymorphism for a range of fixed strategies. The rodent malaria parasite studies argue for a plastic response by *P. chabaudi* with a remarkable ability to monitor and respond to clonal diversity within infections (Reece *et al.* 2008). The *P. gallinaceum* studies show that parasite monitors changes in the hormonal levels of its bird hosts that allows sex ratio adjustment based on level of immune attack (Paul 2000). The *P. mexicanum* experiments have the advantage of using a natural parasite-host system, but the disadvantage of lacking well-characterized clones of parasite to induce infections in hosts with minimal genetic variation. Our results are therefore equivocal. *P. mexicanum* may have phenotypic

plasticity for sex ratio but produce so few gametes per male gametocyte that even single-clone infections produce close to 50:50 sex ratios, or the parasite may follow a fixed strategy of fertility insurance because of factors that consistently severely limit male gamete survival or mobility, such as quickly clotting blood. A fixed sex ratio near 50% male may also be favoured because multi-clone infections are common (Vardo and Schall, 2007).

Sex ratio theory is among the most productive and successful programs in evolutionary biology, and reveals how selection acting on individual organisms leads to the variation in sex ratios seen in populations (Charnov, 1982). This theory has now been usefully applied to malaria parasites, suggesting a central feature of the parasites' life history can now be understood (Read *et al.* 1992; Reece *et al.* 2003, 2008; West *et al.* 2001, 2002). Studies across species with different hosts and evolutionary histories, such as *P. mexicanum*, are necessary before making any general conclusions on allocation of gametocytes to male and female cells.

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References

- Alano, P. and Billker, O.** (2005). Gametocytes and Gametes. In *Molecular Approaches to Malaria* (ed. Sherman, I. W.), pp. 191-219. ASM Press, Washington DC.
- Bromwich, C. R. and Schall, J. J.** (1986). Infection dynamics of *Plasmodium mexicanum*, a malarial parasite of lizards. *Ecology* **67**, 1227-1235.
- Burkot, T. R., Williams, J. L., and Schneider, I.** (1984). Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 339-341.
- Carter, R., R. W. Gwadz, and F. M. McAuliffe.** (1979) *Plasmodium gallinaceum*: Transmission-blocking immunity in chickens. I. Comparative immunogenicity of gametocyte- and gamete-containing preparations. *Experimental Parasitology* **47**, 185-193.
- Charnov, E. L.** (1982) *The Theory of Sex Allocation*. Princeton University Press, Princeton, New Jersey, 355 pp.
- Eisen, R. J. and Schall, J. J.** (2000). Life history of a malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *Proceedings of the Royal Society of London* **267**, 793-799.
- Fialho, R. F. and Schall J. J.** (1995) Thermal ecology of a malarial parasite and its insect vector: Consequences for the parasite's transmission success. *Journal of Animal Ecology* **64**, 553-562.
- Gardner, A., Reece, S. E. and West, S. A.** (2003). Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *Journal of Theoretical Biology* **223**:515-521.
- Garnham, P. C. C.** (1966). *Malaria parasites and Other Hemosporidia*. Blackwell Scientific Publications, Oxford, UK, 1114 pp.
- Hamilton, W. D.** (1967) Extraordinary sex ratios. *Nature* **156**, 477-488.

- Osgood, S. M., Eisen, R. J., Wargo, A. R., and Schall, J. J.** (2003). Manipulation of the vertebrate host's testosterone does not affect gametocyte sex ratio of a malaria parasite. *Journal of Parasitology* **89**, 190-192.
- Osgood, S. M. and Schall, J. J.** (2004). Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* **128**, 23-29.
- Paul, R. E. L., Coulson, T. N., Raibaud, A., and Brey, P. T.** (2000). Sex determination in malaria parasites. *Science* **287**, 128-131.
- Paul, R. E.** (1999). Sex ratio adjustment in *Plasmodium gallinaceum*. *Parassitologia* **41**, 153-158.
- Pickering, J., Read, A. F., Guerrero, S., and West, S. A.** (2000). Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research* **2**, 171-184.
- Read, A. F., Narara, A., Nee, S., Keymer, A. E., and Day, K. P.** (1992). Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* **104**, 387-395.
- Reece, S. E., Drew, D. R., and Gardner, A.** (2008). Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**, 609-614. doi:10.1038/nature06954
- Reece, S. E., Duncan, A. B., West, S. A., and Read, A. F.** (2003). Sex ratios in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* **127**, 419-425.
- Robert, V., Sokhana, C. S., Rogier, C., Ariey, F., and Trape, J.-F.** (2003). Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* **127**, 1-8.
- Schall, J. J.** (1996) Malarial parasites of lizards: diversity and ecology. *Advances in Parasitology* **37**, 255-333.
- Schall, J. J.** (1989). The sex ratio of *Plasmodium* gametocytes. *Parasitology* **98**, 343-350.
- Schall, J. J.** (1990) Virulence of lizard malaria: The evolutionary ecology of an ancient parasite-host association. *Parasitology* **100**:S35-S52.
- Schall, J. J.** (2000). Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* **121**, 575-580.

- Schall, J. J.** (2002). Parasite virulence. In *The Behavioural Ecology of Parasites* (ed. Lewis, E., Campbell, J. and Sukhdeo, M.) pp. 283-313. CAB International, Oxon, UK.
- Schall, J. J.** (2009). Do malaria parasites follow the algebra of sex ratio theory? *Trends in Parasitology* **25**, 120-123.
- Schall, J. J. and Vardo, A. M.** (2007). Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Molecular Ecology Notes* **7**, 227-229.
- Taylor, L. H. and A. F. Read** (1997) Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitology Today* **13**, 135-140.
- Vardo, A. M. and Schall, J. J.** (2007). Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Molecular Ecology* **13**, 2712-2720.
- Vardo-Zalik, A. M. and Schall, J. J.** (2008). Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* **135**, 1363-1372.
- Vardo-Zalik, A. M. and Schall, J. J.** (2009). Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* **90**(2), 529-536.
- West, S. A., Reece, S. E., and Read, A. F.** (2001). Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology* **17**, 525-531.
- West, S. A., Smith, T. G., Nee, S., and Read, A. F.** (2002). Fertility insurance and the sex ratios of malaria and related hemosporidian blood parasites. *Journal of Parasitology* **88**, 258-263.
- Wilson, K. and Hardy, I. C. W.** (2002) Statistical analysis of sex ratios: an introduction. In *Sex Ratios: Concepts and Research Methods* (ed. Hardy, I. W. C.), pp. 48-92. Cambridge University Press, Cambridge.

**CHAPTER 3: Male gametocyte fecundity and sex ratio of a malaria parasite,
*Plasmodium mexicanum***

Allison T. Neal

Summary

Evolutionary theory predicts that the sex ratio of *Plasmodium* gametocytes will be determined by the number of gametes produced per male gametocyte (male fecundity), parasite clonal diversity and any factor that reduces male gametes' ability to find and combine with female gametes. Despite the importance of male gametocyte fecundity for sex ratio theory as applied to malaria parasites, few data are available on gamete production by male gametocytes. In this study, exflagellating gametes, a measure of male fecundity, were counted for 866 gametocytes from 26 natural infections of the lizard malaria parasite, *Plasmodium mexicanum*. The maximum male fecundity observed was 8, but most gametocytes produced 2-3 gametes, a value consistent with the typical sex ratio observed for *P. mexicanum*. Male gametocytes in infections with higher gametocytaemia had lower fecundity. Male fecundity was not correlated with gametocyte size, but differed among infections, suggesting genetic variation for fecundity. Fecundity and sex ratio were correlated (more female gametocytes with higher fecundity) as predicted by theory. Results agree with evolutionary theory, but also suggest a possible tradeoff between production time and fecundity, which could explain the low fecundity of this species, the variation among infections, and the correlation with gametocytaemia.

Introduction

Sex ratio theory is a venerable focus in evolutionary biology originating with Darwin's verbal treatment and a mathematical model presented by Düsing (reviewed in Edwards, 1998). The application of the theory to sexually reproducing protists such as malaria parasites (*Plasmodium* and related genera, Martinsen *et al.*, 2008), though, is much more recent (Godfray and Werren, 1996; Read *et al.*, 1992; Schall, 2008; Schall, 2009). Read *et al.* (1992) recognized that one model in sex ratio theory, Local Mate Competition (LMC, Hamilton, 1967) fits the breeding structure of malaria parasites and can account for the variation in gametocyte sex ratio seen among malaria parasite species, among geographic populations within a species, and even within individual infections over time (Schall, 2009). This model concludes that the unbeatable sex ratio of populations with little genetic diversity will be strongly female biased (with the specific bias dependent on the degree of inbreeding), but that this bias may be limited by male fecundity. The proportion of males in a population should have a minimum of $1/(1 + c)$, where c is male fecundity, to ensure all females are mated. This minimum is particularly important in predicting the sex ratio of clonal populations, such as single-genotype infections of *Plasmodium*, because there should be just enough males to mate with all females when inbreeding is complete. Despite the importance of fecundity for sex ratio theory, detailed data on gamete production in *Plasmodium* parasites are scarce.

The sexual cells (gametocytes) of malaria parasites are produced following bouts of asexual replication in the vertebrate host and remain in the host's erythrocytes undergoing no further development until they are taken up by a blood-feeding insect vector (Garnham, 1966). Within the insect midgut, these sexually dimorphic

gametocytes begin rapid gametogenesis. Each female gametocyte produces a single large gamete. The haploid male genome replicates 3 times to produce a maximum of 8 gametes (Alano and Billker, 2005), although gamete production can be lower if the process is truncated or if anucleate gametes are produced (Sinden, 1983; Sinden *et al.*, 1978). The number of gametes produced by a male gametocyte (male fecundity) must be a life history trait that is critical for fertilization and transmission success in addition to its predicted importance in shaping gametocyte sex ratio.

Though there are few data on male gametocyte fecundity related to sex ratio, several perplexing relevant observations emerge from data on the sex ratios of various *Plasmodium* species. First, the gametocyte sex ratios of *P. mexicanum* and other lizard malaria parasites tend to be far less female biased than seen in the malaria parasites of birds and mammals (Schall, 2009), even for single-clone infections (Neal and Schall, 2010). Low male gametocyte fecundity in *P. mexicanum* and other reptilian malaria parasites is one possible explanation for less female-biased sex ratios. If variation in fecundity among taxa exists, why should such an important life history trait vary, especially in a way that could reduce fertilization and transmission success? Second, gametocyte sex ratio appears to differ among parasite genotypes for *P. mexicanum* (Neal and Schall, 2010), for the rodent malaria parasite *P. chabaudi*, (Reece *et al.*, 2008) and for *P. falciparum* (Burkot *et al.*, 1984; Ranford-Cartwright *et al.*, 1993). Variation in fecundity within a species, like variation among taxa, is a possible cause of variation in sex ratio; if so, this again begs the question of why there would be a genetic polymorphism for such an important life history trait.

To cast light on this issue, I gathered data on the male fecundity and gametocyte sex ratio, size and density from natural infections of the lizard malaria parasite *P. mexicanum*. My goals were: (1) To determine the fecundity of *P. mexicanum*. The sex ratios of single-clone infections of *P. mexicanum* are generally around 40% male (Neal and Schall, 2010), suggesting that the fecundity of this parasite is low. If the parasite conforms to the LMC model, a sex ratio of 40% male would predict a male fecundity of only 1 - 2. (2) To determine whether fecundity is related to other infection traits such as gametocyte size, the clonal diversity of the infection, asexual parasitaemia, or gametocytaemia. Schall (1989) found evidence that gametocytes in natural infections increase in size over time, so larger gametocytes may have more energy to invest in gamete production, or their large size might be due to swelling associated with senescence. Also, variation in fecundity might provide an alternative approach to fertility insurance (West *et al.*, 2001). Fertility insurance predicts an increase in the proportion of male gametocytes in response to conditions that would reduce male gamete success to insure fertilization. A similar increase in the total number of male gametes could be achieved by increasing the number of gametes produced per male gametocyte. This could lead to variation in fecundity due to competition with other clones, interactions with the immune system (possibly related to asexual parasitaemia), or male gametes' ease of finding females (related to gametocytaemia). (3) To test whether there is variation among infections for male gametocyte fecundity and whether it is correlated with sex ratio in single-clone infections. Neal and Schall (2010) found evidence of genetic variation for sex ratio, which could be caused by genetic variation for male fecundity. If

this is the case, fecundity should vary among infections and be correlated with the sex ratios of single-clone infections.

Materials and Methods

Infected lizards

The infections of *P. mexicanum* used for this study were all sampled from naturally infected western fence lizards (*Sceloporus occidentalis*) captured during July – August 2009 at the University of California Hopland Research and Extension Center, located in Mendocino County, California, USA (Fricke *et al.*, 2010). Drops of blood were taken from each lizard to produce a thin blood smear for Giemsa staining and dried and frozen drops on filter paper. Lizards harbouring gametocytes were identified by scanning the stained smears at 1000 x. Infected lizards were housed outside in vector-proof cages for three days during which time 2 μ L blood was drawn daily for exflagellation experiments. The lizards were subsequently released at their site of capture.

Male gametogenesis

Lizards were kept in an incubator at 34°C (the lizards' preferred body temperature, Schall, 1990) for at least 30 minutes prior to inducing gametogenesis. Exflagellation was induced by mixing 2 μ L blood with 6 μ L of exflagellation medium (50 mM NaHCO₃) on a slide and covering it with a cover slip. Other exflagellation media were tested in preliminary experiments, most of which contained combinations of glucose, Na⁺, and Cl⁻, and some additionally contained xanthurenic acid (Alano and

Billker, 2005). Most combinations were not successful at inducing exflagellation, and none were as effective as the simple NaHCO₃ solution (data not shown). Within minutes after mixing blood with the medium, the gametocytes rounded up into spheres similar to that seen in other *Plasmodium* species (Carter and Graves, 1988). Exflagellating gametocytes were first observed 20 minutes after the blood was combined with exflagellation medium and the highest proportion of gametocytes exflagellating occurred after about 25-35 minutes. Therefore slides were kept on a damp filter paper in a Petri dish for 25 min before observations began for counting gametes. I examined the preparations at 1000 x for 10 minutes, and after locating each exflagellating gametocyte, I counted the number of flagella emerging and assumed that any gametocyte that was exflagellating was mature. I estimated the exflagellation rate of each infection by dividing the number of exflagellating gametocytes observed during this time period by the total number of gametocytes observed during this time. I repeated this procedure on three consecutive days for each infected lizard and varied the time of day that the gamete counts were performed to ensure that there was no variation in fecundity on a small time scale.

Clonal diversity within infections

To determine the genetic diversity of each infection, I extracted DNA from the blood dots on filter paper using a Qiagen DNEasy blood and tissue kit (Qiagen Sciences, Germantown, Maryland, USA). Samples were subjected to PCR for 4 microsatellite loci (Pmx 306, 732, 747, and 839) using primers and conditions presented by Schall and Vardo (2007). Anderson *et al.* (2010) showed that 4 loci could differentiate

approximately 90% of genetic clones for *Plasmodium falciparum*. The primers used amplify DNA from both asexual and sexual stage parasites, but Vardo-Zalik (2009) showed that a high proportion of alleles (and thus clones) and their relative proportions detected using this method can later be found in the DNA of oocysts in the insect vector, suggesting that this method is useful for estimating the genetic diversity of the sexual cells alone. The PCR product was analyzed using an ABI prism genetic analyzer and the results were viewed using the program GeneMapper (ABI, Carlsbad, California, USA). Because all stages of *P. mexicanum* found in the lizard are haploid, I scored an infection as having a single clone if only 1 allele was seen at each of the 4 loci. Multi-clone infections had at least 2 alleles for at least 1 of the loci.

Gametocyte sex ratio

To determine the sex ratio of each single-clone infection, stained slides were viewed at 1000 x on a light microscope. Male and female gametocytes can be readily distinguished based on differences in staining as described by Schall (1989). Mature gametocytes were scored as male or female until 200 gametocytes were scored. Care was taken to view many fields from all areas of each slide to reduce the effect of possible aggregation of gametocytes. When there were > 30 gametocytes per 1000 erythrocytes, avoiding possible aggregates was problematic, so these sex ratio counts ($n = 2$) were excluded from sex ratio analysis, though the infections were included in all other analyses.

The equation $1/(1+c)$, where c is male fecundity, represents the minimum sex ratio (proportion of males) expected by LMC for any infection, and therefore the

predicted sex ratio of single-clone infections. I used this equation to calculate the predicted sex ratio of each single-clone infection by using a summary statistic describing the average fecundity of each infection (λ , described in more detail below) as an estimate of c in the equation above. Since previous studies have shown that a high proportion of gametes produced may be dysfunctional (Sinden, 1983; Sinden *et al.*, 1978), I repeated this calculation assuming that on average only 40% or 77% of gametes were viable [the values observed by Sinden (60% anucleate, 1983) and Sinden *et al.* (23% anucleate, 1978)]. I used linear regression analysis to compare predicted sex ratios with the sex ratio counts for all infections.

Male gametocyte size and gametocytaemia

The size of gametocytes prior to the onset of gametogenesis was determined by photographing 30 male gametocytes per infection from stained slides using a Moticam 1000 microscope camera. I measured the area of both the gametocyte and the red blood cell it was infecting using the software Motic Images Plus 2.0 (Motic China Group Co.). Gametocyte size was analyzed in two ways: as the raw gametocyte area and as gametocyte area divided by red blood cell area. The ratio was meant to control for differences in gametocyte size that might have arisen from unknown variation in making smears. Gametocytaemia and asexual parasitaemia were determined by counting 1000 erythrocytes on a stained slide and noting the number of gametocytes or asexual parasites observed therein. Both are expressed here as parasites per 1000 erythrocytes. Slides were viewed at 1000x on a light microscope and care was taken to sample a wide range of locations on the slide.

Statistical analysis

All statistical analysis was performed using JMP 8 (SAS Institute, Cary, NC, USA) except the multiple regression analysis, which was performed using SAS 9.2 (SAS Institute, Cary, NC, USA). When 1.0 was subtracted from the number of gametes produced by each gametocyte, these counts did not differ significantly from a Poisson distribution ($P = 0.298$), so this transformation was used for all analyses.

To compare fecundity with other infection traits, I fit a Poisson distribution to the gamete counts from each infection and determined λ , the measure of central tendency for a Poisson distribution. A multiple regression with backward selection (terms were sequentially removed from the maximal model) tested the effect of logarithmically transformed gametocytaemia, logarithmically transformed asexual parasitaemia, and gametocyte size (corrected and uncorrected, two separate models) on fecundity (λ). A second multiple regression using the same selection method examined the effect of logarithmically transformed gametocytaemia and fecundity (λ) on sex ratio.

I used a generalized linear model (GLM) with Poisson distribution and Log link function to determine if there was variation among infections for the number of gametes produced by each male gametocyte (male fecundity). A second GLM tested if the clonal diversity of an infection (single vs. multiple clones) affected gamete production. The day on which counts were performed (1, 2, or 3) was included as a model effect in both GLM to account for replication. An ANOVA tested for variation in gametocyte size (corrected and uncorrected by erythrocyte area) among infections. I examined the variation in fecundity within infections for single- and multi-clone infections using range (maximum

minus minimum male fecundity) and median absolute deviation (MAD, a robust measure of spread, Hoaglin *et al.*, 1983). When 3 was subtracted from the range, the values did not differ from a Poisson distribution, and thus a GLM analysis was performed.

Results

Distribution of male gametocyte fecundities

Gamete counts were performed for 26 naturally infected lizards for a total of 866 exflagellating gametocytes examined. For one infection, only one exflagellating gametocyte was observed and the infection and its gamete count were therefore excluded from further analysis. The number of exflagellating gametocytes observed for each infection over the course of three days ranged from 7 to 91 with a mean of 34.6. The day on which counts were performed did not affect fecundity ($p > 0.4$, GLM). The median and mode of all gamete counts combined was 3 (range = 1 – 8, $\lambda = 2.69$; Fig. 7). The median gamete counts and λ (shown in Fig. 8 with confidence intervals) for each infection ranged from 2 to 4.

Factors affecting male fecundity

Out of 25 infections, there were 23 unique genotypes based on the 4 microsatellite markers. Two pairs of infections had identical genotypes. There was significant variation among infections for male fecundity ($P < 0.0001$, GLM), but it did not differ for the two pairs of infections with the same genotype ($P > 0.60$, GLM). Fig. 8 shows fecundity (λ) values and their confidence intervals for each infection.

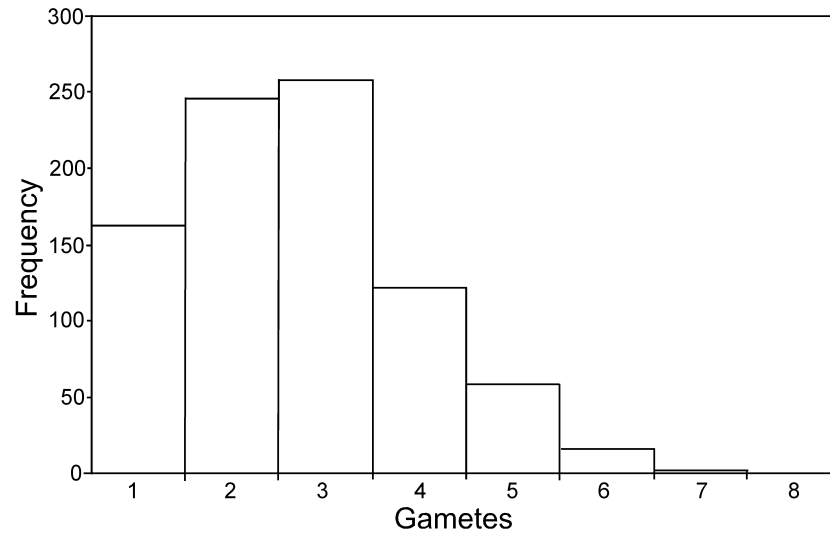


Figure 7: Distribution of gametes produced by each exflagellating male gametocyte for natural *P. mexicanum* infections. This distribution is based on counts of 865 exflagellating gametocytes from 25 natural infections. Data were collected from each infection on three consecutive days at different times of the day.

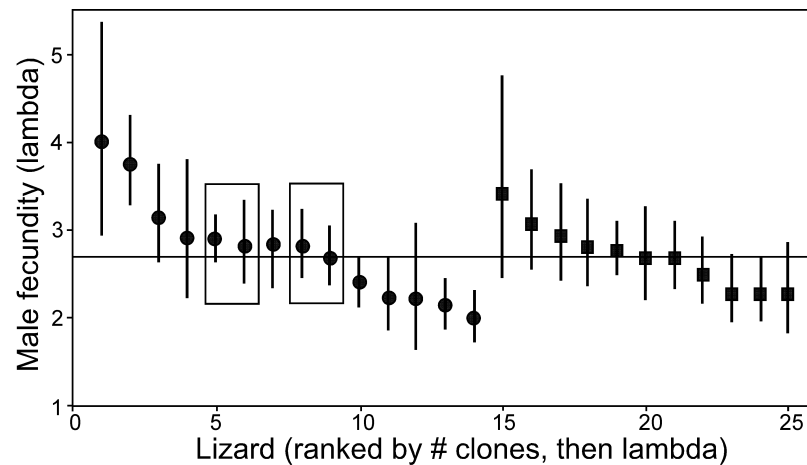


Figure 8: Male gametocyte fecundities (lambda values) of individual natural infections of *Plasmodium mexicanum*. Lambda values were obtained from a Poisson distribution fitted to counts of number of gametes produced by exflagellating gametocytes. Single- (circles) and multi-clone (squares) infections are ranked by lambda. Vertical bars represent a 95% confidence interval around the estimate. Infections in boxes represent pairs of infections with identical alleles for 4 microsatellite loci. The horizontal bar is the lambda of all counts combined.

Gametocytaemia was the only significant predictor of fecundity in the multiple regression model ($R^2 = 0.209$, $P = 0.022$). While asexual parasitaemia (range $<1 - 272$) and gametocytaemia (range $1 - 176$) were significantly correlated ($R = 0.675$, $P =$

0.0002), simple correlation confirms the lack of significant correlation between asexual parasitaemia and fecundity ($R = -0.180$, $P = 0.387$). Male gametocytes in infections with higher gametocytaemia produced fewer gametes (Fig. 9).

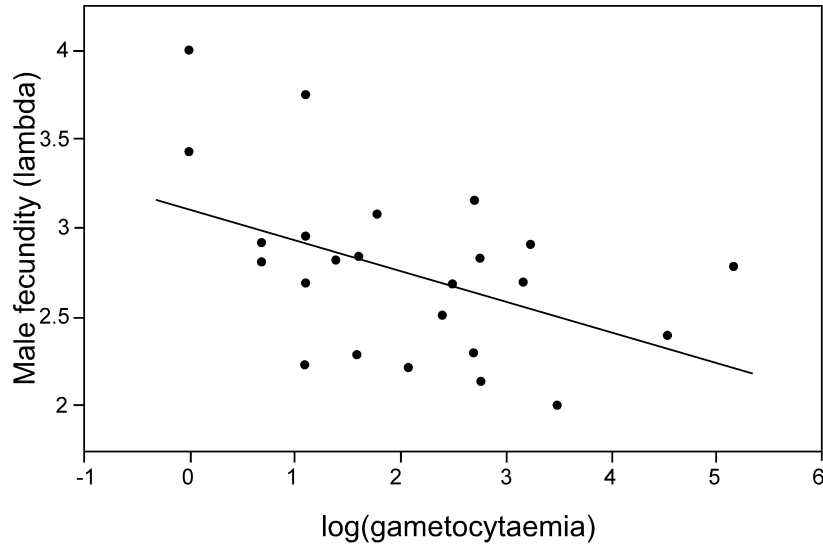


Figure 9: Relation between log transformed gametocytaemia (gametocytes per 1000 erythrocytes) and male gametocyte fecundity (lambda). Each point represents one infection. Line is a regression fit to the data; $P = 0.0216$, $r^2=0.2089$.

When tested separately, both single- and multi-clone infections showed significant variation in fecundity among infections (single: $P < 0.0001$, multi: $P = 0.048$, GLM), though there was no difference in fecundity between the single- and multi-clone infections overall ($P=0.603$, GLM). Also, the range of individual male gametocyte fecundities observed in single- and multi-clone infections did not differ ($P = 0.959$, GLM). The MAD for all infections except 2 (1 single- and 1 multi-clone infection) were 1.0. Because almost no variation existed in MAD of gamete production, no statistical analysis was performed on these values.

There was no relationship between gametocyte size and the exflagellation rate (range 10 - 30%) of the infections ($p > 0.30$, $R^2 < 0.05$, linear regression), and although

both gamete production (above) and gametocyte size ($P < 0.0001$, ANOVA) varied significantly among infections, gametocyte size was not a significant predictor of fecundity (above, multiple regression).

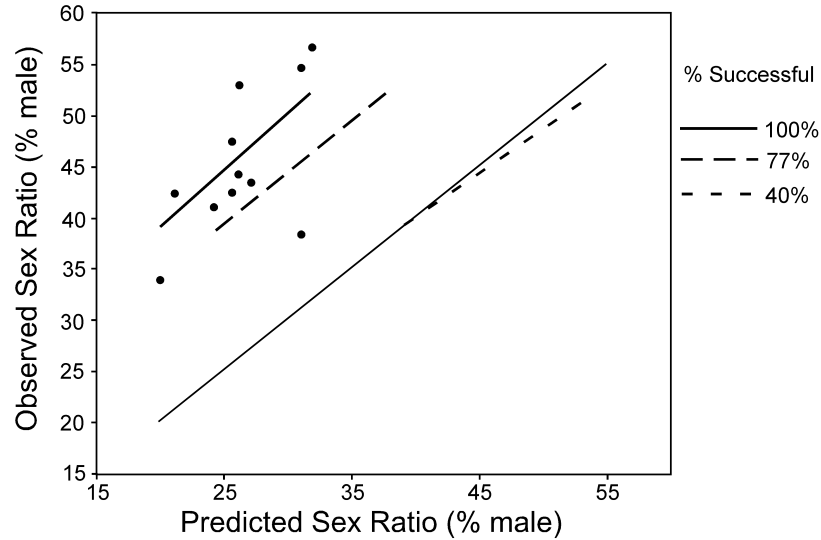


Figure 10: Relation between observed and predicted sex ratio. Sex ratio was predicted using the equation $1/(1+c)$, with average male gametocyte fecundity (λ) as an estimate of c under three assumptions of the percent of gametes that were viable (100%, 77%, and 40%). The thin solid line indicates observed = predicted. Data points for 100% viable gametes are shown with a thick solid line fit to those data. For the other viability percents, only the regression lines are shown. Sex ratios among infections approximate theory (significant regression with slope near predicted $y = x$), and observed sex ratios best fit expected values when the viability of gametes is approximately 40% (average 0.8 – 1.6 viable gametes).

Sex ratio and fecundity

The average sex ratio of single-clone infections in this study was 43.8% male and ranged from 34 – 58% male. Fecundity was the only significant predictor of sex ratio in the multiple regression model ($R^2 = 0.371$, $P = 0.036$). While gametocytaemia and fecundity are correlated ($R = -0.457$, $P = 0.022$), simple correlation confirms the lack of significant correlation between gametocytaemia and sex ratio ($R = -0.073$, $P = 0.804$).

Linear regression showed a significant correlation between predicted and observed sex ratio for all percentages of successful gametes tested (40, 77, 100% successful, $R^2 > 0.34$, $P < 0.05$). The slope of the regression line is similar to the slope of $y = x$ (as expected if observed = predicted) over a range of values for the percentage of successful gametes. If 40% of gametes are successful, there is a close concordance between observed and predicted values (Fig. 10).

Discussion

Although sex ratio theory originated with studies by Darwin and Düsing about the same time that the malaria parasite's life cycle was being revealed (Edwards, 1998; Schall, 2009), the usefulness of selectionist thinking to explain the within- and among-species variation in gametocyte sex ratios was only recognized over the past two decades (Paul *et al.*, 1999; Read *et al.*, 1992; Schall, 1989). In particular, the model of Local Mate Competition has suggested that parasite clonal diversity in the vertebrate host blood (and thus the degree of inbreeding) and male fecundity are central in molding the proportion of male and female gametocytes produced in an infection (Reece *et al.*, 2008; Reece *et al.*, 2003). My study centered on male gametocyte fecundity of the lizard malaria parasite, *P. mexicanum*, to pursue several questions: Is the male fecundity of *P. mexicanum* low (1 – 2) as expected under the LMC model based on the only slightly female biased gametocyte sex ratio seen in single-clone infections of this species? Is there genetic variation for male fecundity as suggested by the apparent genetic variation in gametocyte sex ratio for this species? Does male fecundity vary based on gametocyte density, asexual parasite density, gametocyte size, or infection clonal diversity? Do male

fecundity and gametocyte sex ratio covary among infections as predicted by sex ratio theory? The results reported here suggest that fecundity is low, as expected, and varies among infections. Further, fecundity was found to be correlated with gametocytaemia, and the relationship between the fecundity and sex ratio of single-clone infections is very similar to the prediction of LMC.

Male gametocyte fecundity

Studies on *P. falciparum* show that the parasite undergoes three nuclear divisions within the male gametocyte, leading to a maximum of 8 male gametes (Alano and Carter, 1990; Lobo and Kumar, 1998; Sinden, 1985). *Plasmodium mexicanum* produced a maximum of 8 male gametes, but this was uncommon. Most male gametocytes produced 2-3 gametes, suggesting that a large proportion of male gametocytes do not achieve their maximal potential fecundity. Reece *et al.* (2008) found that male gametocytes of a clone of *P. berghei* produce a mean of 2.03 viable gametes and Ranford-Cartwright (1995) estimated that two clones of *P. falciparum* both produced an average of 4-5 gametes per gametocyte. The data from *P. mexicanum* presented here is additional support for the possibility that male gametocytes often fail to achieve their maximal potential fecundity, and combined with data from other species referenced above, suggests that there may be variation in fecundity among species.

Also, I found variation in fecundity among the infections used in this study. While it is unclear from the data presented here whether this variation was caused by primarily genetic or environmental factors, Neal and Schall's (2010) study found a donor

effect on sex ratio in replicate single-clone infections suggestive of genetic variation for gametocyte sex ratio, which could be explained if there is genetic variation for fecundity.

Fecundity and other infection traits

The only factor measured in this study that appears to affect male fecundity is gametocytaemia: infections with a lower density of gametocytes tend to have higher male fecundity. If there is a tradeoff between fecundity and speed of gamete production (discussed below), low gametocytaemia may select for higher fecundity at the expense of speed to ensure that there are sufficient male gametes to locate the rare female gametes in the mating environment. Adjusting fecundity in response to low gametocytaemia could be an alternative to the sex ratio shift traditionally predicted by Fertility Insurance, and would result in a similar increase in the proportion of male gametes available for mating. If fecundity adjustment is a means of Fertility Insurance, fecundity might also be predicted to correlate with asexual parasitaemia, since asexual parasitaemia may often be related to an immune response. It is asexual parasites that often trigger an immune response, and immune factors carried over from the lizard could kill male gametes in the sand fly. However, I found no evidence of such a relationship. It is possible that an immune response, if mounted, would affect gamete success more than gamete production and therefore not be detectable in a study that measures only gamete production. Also, little is known about the response of the lizard immune system to *P. mexicanum*, but data (summarized by Neal and Schall, 2010) suggest the immune response is weak or non-existent.

Competition for mating success might also be predicted to cause differences in fecundity between single- and multi-clone infections if fecundity is plastic, or a greater range in fecundity in multi-clone infections if fecundity is fixed but varies among genotypes. However, no difference in fecundity or range of fecundity between single- and multi-clone infections was observed.

Gametocyte size, which varies among infections, was also found to be unrelated to both fecundity and exflagellation rate. Schall (1989) observed that gametocytes of *P. mexicanum* increase in size over the course of natural infection, but larger gametocytes have no increased mating potential based on the factors measured here.

Sex ratio and fecundity

The average sex ratio of single-clone infections in this study (43.8% male) was almost identical to the sex ratio reported by Neal and Schall (2010) for experimentally induced infections and Schall (1989) for natural infections. Sex ratio was associated with fecundity in single-clone infections, and the sex ratios predicted under LMC using these fecundities fit the observed values well. If only about 40% of gametes are successful on average (due to deformation or factors associated with fertility insurance), the regression closely approximates the line $y = x$. This concordance with evolutionary theory is striking because the variation in both gametocyte sex ratio and fecundity was not great among infections and argues that selection must be intense on molding gametocyte sex ratio in malaria parasites (Reece *et al.*, 2008). Additionally, the assumption of 40% successful gametes is consistent with Sinden's (1983) observation that up to 60% of

gametes produced are not functional, suggesting that such low gamete success is plausible and perhaps even likely.

Low fecundity: constrained or adaptive

Male fecundity appears low for *P. mexicanum*, and may drive gametocyte sex ratio toward a higher proportion of male cells than observed in other *Plasmodium* species, including the human malaria parasites (Schall, 2009). Why should fecundity, which must be an important life history trait, be so often reduced below the maximum possible of 8 gametes in this species? It seems that low fecundity must either be due to constraint, meaning that gametocytes are unable to produce more gametes given certain conditions, or be adaptive.

Possible constraints on gametogenesis include resource limitation, gametocyte maturity and time. Alano and Billker (2005) propose a tradeoff between speed and quantity in gamete production such that gametocytes that rapidly produce gametes are not able to consistently or accurately produce a maximum number. Thus time constraints placed on gametogenesis by removal of water from the blood meal in the insect's midgut (Alano and Billker, 2005) or the rapid clotting of blood seen in fence lizards infected with *P. mexicanum* (Vardo-Zalik and Schall, 2008) could select for faster gamete production and thus lower fecundity.

Adaptive reductions in fecundity may also be feasible, particularly if a tradeoff between speed and quantity of gamete production exists. Some conditions might favor the quick production of fewer gametes (such as competition among clones), whereas others might favor slower production of more gametes (such as infections with low

gametocytaemia, above). Reduction in fecundity could be facultative, allowing the parasite to respond to immediate conditions, or fixed, for example if genes that tend to cause high gametocytaemia and low fecundity travel together.

The results presented here are contradictory: the correlation between gametocytaemia and fecundity found in this study suggests that low fecundity may be, at least in part, adaptive, but there was no relationship between the clonal diversity and fecundity. It is possible that low fecundity is adaptive but not plastic, however further investigation is necessary before a more definitive conclusion can be reached. Knowing whether fecundity and timing of gametogenesis are in fact related, and how the length of gametogenesis relates to the time available to the parasite in the sand fly could help determine if low fecundity is due to a time constraint. If low fecundity is adaptive, it would be interesting to vary environmental conditions such as gametocytaemia and determine whether the parasite shifts its fecundity in response.

Potential sources of error in measures of male fecundity

The number of flagella emerging during exflagellation was used here to estimate male gametocyte fecundity. Since the in vitro environment in which this study was carried out is quite different from a sand fly midgut, some care should be taken in interpreting these results. Also, several sources of error could bias these results. Sinden *et al.* (1978) reported that gametes of *P. falciparum* are not always released synchronously. If this is also true for *P. mexicanum*, some gametes may have already left a gametocyte when I examined it. Also, I did not stain each gamete for a nucleus, and could not determine the proportion of gametes that were not functional. Though these

factors could bias the fecundity estimates, they would do so in opposing directions. Gametes that were not nucleated but were counted would lead to an overestimate of fecundity, whereas gametes that had already escaped and consequently were not counted would lead to an underestimate of fecundity. For the study of variation among infections, all infections should have been affected by these factors randomly, and thus should not affect the interpretation of the results on variation in fecundity.

Conclusion

The observed patterns connecting male gametocyte fecundity with gametocyte density and sex ratio are striking, especially when several sources of error in counting male gametes and gametocyte sex ratio will result in some degree of experimental error. The results agree with sex ratio theory, both in a broad sense (the low fecundity of males was predicted based on the distribution of gametocyte sex ratios) and within infections (the higher production of male gametocytes in infections with lower fecundity). These findings argue that the sexual stages in the life history of malaria parasites continue to offer intriguing problems for an evolutionary and ecological approach to the study of *Plasmodium* biology.

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References

- Alano, P. and Billker, O.** (2005). Gametocytes and gametes. In *Molecular Approaches to Malaria* (ed. Sherman, I. W.), pp. 191-219. ASM Press, Washington D.C., USA.
- Alano, P. and Carter, R.** (1990). Sexual differentiation in malaria parasites. *Annual Reviews of Microbiology* **44**, 429-449.
- Anderson, T. J. C., Williams, J. T., Nair, S., Sudimack, D., Barends, M., Jaidee, A., Price, R. N. and Nosten, F.** (2010). Inferred relatedness and heritability in malaria parasites. *Proceedings of the Royal Society B* **277**, 2531-2540. doi: 10.1098/rspb.2010.0196.
- Burkot, T. R., Williams, J. L. and Schneider, I.** (1984). Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from same isolate. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 339-341.
- Carter, R. and Graves, P. M.** (1988). Gametocytes. In *Malaria: Principles and Practice of Malariology* (ed. Wernsdorfer, W. H. and McGregor, I.), pp. 253-305. Churchill Livingstone, Edinburgh, UK.
- Edwards, A. W. F.** (1998). Natural selection and the sex ratio: Fisher's sources. *The American Naturalist* **151**, 564-569.
- Fricke, J. M., Vardo-Zalik, A. M. and Schall, J. J.** (2010). Geographic genetic differentiation of a malaria parasite, *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*. *Journal of Parasitology* **96**, 308-313.
- Garnham, P. C. C.** (1966). *Malaria Parasites and Other Haemosporidia*, Blackwell Scientific Publications, Oxford, UK.

Godfray, H. C. J. and Werren, J. H. (1996). Recent developments in sex ratio studies. *Trends in Ecology and Evolution* **11**, 59-63.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Nature* **156**, 477-488.

Hoaglin, D. C., Mosteller, F. and Tukey, J. W. (1983). Introduction to more refined estimators. In *Understanding Robust and Exploratory Data Analysis* (ed. Hoaglin, D. C., Mosteller, F. and Tukey, J. W.), pp. 283-296. John Wiley & Sons, Inc., New York, USA.

Lobo, C. A. and Kumar, N. (1998). Sexual differentiation and development in the malaria parasite. *Parasitology Today* **14**, 146-150.

Martinsen, E. M., Perkins, S. L. and Schall, J. J. (2008). A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* **47**, 261-273.

Neal, A. T. and Schall, J. J. (2010). Gametocyte sex ratio in single clone infections of the malaria parasite *Plasmodium mexicanum*. *Parasitology* **137**, 1851-1859.

Paul, R. E. L., Raibaud, A. and Brey, P. T. (1999). Sex ratio adjustment in *Plasmodium gallinaceum*. *Parassitologia* **41**, 153-158.

Ranford-Cartwright, L. C. (1995). Fit for fertilization: mating in malaria parasites. *Parasitology Today* **11**, 154-157.

Ranford-Cartwright, L. C., Balfe, P., Carter, R. and Walliker, D. (1993). Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology* **107**, 11-18.

Read, A. F., Narara, A., Nee, S., Keymer, A. E. and Day, K. P. (1992). Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* **104**, 387-395.

Reece, S. E., Drew, D. R. and Gardner, A. (2008). Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**, 609-614. doi: 10.1038/nature06954.

Reece, S. E., Duncan, A. B., West, S. A. and Read, A. F. (2003). Sex ratios in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* **127**, 419-425.

Schall, J. J. (1989). The sex ratio of *Plasmodium* gametocytes. *Parasitology* **98**, 343-350.

Schall, J. J. (1990). Virulence of lizard malaria: The evolutionary ecology of an ancient parasite-host association. *Parasitology* **100**, S35-S52.

- Schall, J. J.** (2008). Sex ratios writ small. *Nature* **453**, 605-606.
- Schall, J. J.** (2009). Do malaria parasites follow the algebra of sex ratio theory? *Trends in Parasitology* **25**, 120-123. doi:10.1016/j.pt.2008.12.006.
- Schall, J. J. and Vardo, A. M.** (2007). Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Molecular Ecology Notes* **7**, 227-229.
- Sinden, R. E.** (1983). Sexual development of malaria parasites. *Advances in Parasitology* **22**, 154-216.
- Sinden, R. E.** (1985). Gametocytogenesis in *Plasmodium spp.* and observations on the meiotic division. *Annales de la Societe belge de médecine tropicale* **65**, Suppl. 2 21-23.
- Sinden, R. E., Canning, E. U., Bray, R. S. and Smalley, M. E.** (1978). Gametocyte and gamete development in *Plasmodium falciparum*. *Proceedings of the Royal Society B* **201**, 375-399.
- Vardo-Zalik, A. M.** (2009). Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host. *International Journal for Parasitology* **39**, 1573-1579.
- Vardo-Zalik, A. M. and Schall, J. J.** (2008). Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* **135**, 1363-1372.
- West, S. A., Reece, S. E. and Read, A. F.** (2001). Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology* **17**, 525-531.

CHAPTER 4: Testing sex ratio theory with the malaria parasite *Plasmodium mexicanum* in natural and experimental infections

Allison T. Neal and Joseph J. Schall

Abstract

The malaria parasite (*Plasmodium*) life history accords well with the assumptions of Local Mate Competition (LMC) of sex ratio theory. Within a single meal of the blood-feeding vector, sexually dimorphic gametocyte cells produce gametes (females produce 1, males several) that mate and undergo sexual recombination. The theory posits several factors drive the *Plasmodium* sex ratio: male fecundity (gametes/ male gametocyte), number and relative abundance of parasite clones, and gametocyte density. We measured these traits for the lizard malaria parasite, *P. mexicanum*, with a large sample of natural infections and infections from experiments which manipulated clonal diversity. Sex ratio in single-clone infections was slightly female-biased, but matched predictions of theory for this low-fecundity species. Sex ratio was less female-biased in clonally diverse infections as predicted by LMC for the experimental, but not natural infections. Gametocyte density was not positively related to sex ratio. These results are explained by the *P. mexicanum* life history of naturally low clonal diversity and high gametocyte production. This is the first study of a natural malaria system that examines all traits relevant to LMC in individual vertebrate hosts and suggests a striking example of sex ratio theory having significance for human public health.

Introduction

When founding the study of sex ratio evolution, Darwin lamented the paucity of cross-taxa data that are required to reveal general patterns; in short, he found “the materials are scanty” (Darwin 1871). Today there is no shortage of such data, and sex ratio theory has developed into one of the most successful and productive research programs in evolutionary biology (Charnov 1982; West 2009). However, until recent years, research has centered primarily on the sex ratios of large, charismatic organisms such as plants and animals, with little notice given to sexually reproducing protists, the dioecious single-celled eukaryotes. Ghiselin (1974) long ago noted that these protists, with their diverse life histories, should offer unexpected insights into the generality of life history theory. We focus here on one example, the malaria parasites (*Plasmodium* and related genera in the phylum Apicomplexa, *sensu* Martinsen et al. 2008).

The life cycle of *Plasmodium*, including the process of sexual recombination by dimorphic cells, was understood by 1910 (Garnham 1966). This was years after Darwin’s brief discussion of sex ratio evolution (1871), but also after Düsing (1884) presented a clear mathematical treatment that introduced the modern theory of sex ratios (translation in Edwards 2000). Sex ratio theory, though, was not applied to malaria parasites until many decades later (Schall 1989; Read et al. 1992). At that time, very few data on sex ratios of malaria parasites had been published (Schall 1989); thus, the materials were again scanty. Several studies have now reported on sex ratios of the malaria parasite sex cells and find variation among parasite species, among populations at different sites, among infections at any site, and even within infections over time

(reviewed in Schall 2009). The origin of this variation begs an explanation, and that explanation should emerge from sex ratio theory.

Plasmodium parasites replicate asexually in the blood of a vertebrate host (bird, squamate reptile, or mammal) over multiple cycles. An infected host may harbor one to several genetically distinct clones of parasite (Read and Day 1992; Paul and Day 1998; Vardo and Schall 2007). Some cells exit the cycle of asexual replication and develop into male and female sexual cells, the gametocytes, which undergo no further reproduction in the vertebrate's blood. The parasites are transmitted between hosts by blood-feeding insect vectors; within minutes of blood ingestion, male and female gametocytes form gametes that mate, creating an ephemeral diploid zygote (all other stages are haploid). Thus, all mating takes place between the gametocytes within a single blood meal. Female gametocytes produce a single female gamete, and males produce up to 8 flagellated male gametes (the maximum based on 3 rounds of mitosis; Alano and Carter 1990; Lobo and Kumar 1998). We refer to the number of gametes produced by a male gametocyte as male fecundity. Meiosis followed by further asexual replication results in haploid transmission-stage parasites (sporozoites) that migrate to the insect's salivary glands.

The first use of sex ratio theory in the study of malaria parasites (Schall 1989) followed Düsing's conclusion that the equilibrium after selection will show equal proportions of males and females. However, Read et al. (1992) recognized that the brief mating in the vector between gametocytes taken from a single vertebrate host matches the population structure assumed for Hamilton's (1967) Local Mate Competition (LMC) model. LMC predicts female-biased sex ratios will be favored by selection when a

population is divided into local breeding patches containing the offspring of only one or a few mothers. For malaria parasites, these breeding patches are the blood meals taken by vectors. Testing LMC for malaria parasites requires accurate counts of male and female gametocytes in the vertebrate host's blood, measures of male gametocyte fecundity, assessments of clone numbers within the host and their relative abundance, and measures of factors that may limit fertilization success (below). However, few empirical studies with malaria parasites to date have measured all the relevant factors because gathering the required data has proven challenging. For example, tests using the *Plasmodium* species infecting humans are hindered by a striking feature of the life histories of these parasites: they typically produce few gametocytes in the blood, preventing reliable sex ratio measures for most individual infections (Taylor and Read, 1997). Other natural systems in which gametocyte density is higher lack data on fecundity and molecular techniques for detailed comparisons of clone numbers and sex ratio (e.g. *Leucocytozoon* spp. [Read et al. 1995] and *Haemoproteus* spp. [Shutler et al. 1995]). The most detailed and elegant tests of the predictions of the LMC model use a rodent malaria parasite of African thick-knee rats in laboratory mice (Reece et al. 2008). Ideally though, evaluations of the theory should examine natural parasite-host associations that have coevolved.

Here we present two studies of gametocyte sex ratio of *Plasmodium mexicanum*, a parasite of the western fence lizard (*Sceloporus occidentalis*) in northern California. This parasite produces large numbers of gametocytes in the lizard host's blood, which allows reliable counts of males and females for most infections. Thus, we were able to gather data on both gametocyte sex ratio and density of the gametocytes in the blood (which likely affects fertilization rates). Using microsatellite markers, we obtained a direct

estimate of number of clones present as well as their relative abundance. We also have an estimate of the male fecundity of *P. mexicanum* (Neal 2011). In the first study, we measured sex ratio and clonal diversity for a large series of naturally infected lizards sampled over three warm seasons. In the second study, we used infections initiated for two experiments in which clonal diversity was experimentally manipulated in the natural lizard host, and gathered similar data for all replicate recipient infections. This is therefore the first report that measures all the relevant factors highlighted by sex ratio theory for a natural *Plasmodium* system: accurate measures of gametocyte sex ratio, male fecundity, number of clones and their relative abundance and gametocyte density.

The Model

The LMC model notes that when only brothers and sisters are present in a patch, a mother can maximize her reproductive output by producing just enough sons to mate with all of her daughters (Hamilton 1967). If more mothers contribute offspring to a patch, then the mothers must balance two competing selective forces: producing more daughters increases the reproductive output of the patch (the number of zygotes), but producing more sons increases fitness relative to others in the patch (as long as males are the less common gender) because on average each son will obtain more mates than each daughter (Taylor and Bulmer 1980; Wilson and Colwell 1981; Frank 1986). Balancing these competing selective pressures yields an expected equilibrium sex ratio of $r = (N - 1)/2N$, where N is the number of mothers depositing equal numbers of offspring in the patch. This relationship can also be expressed in terms of the probability of selfing, s (or, equivalently, the coefficient of inbreeding, F [Dye and Godfray 1993]): $r =$

$(1 - s)/2$ (Read et al. 1992). Note that the critical factor is the likelihood of selfing, which could be high even in multi-clone infections if one clone predominates. If this sex ratio does not result in enough males to mate with all females, the minimum $r_{min} = 1/(1 + c)$, where c is the male fecundity (the number of females each male can mate), is favored instead (Hamilton 1967). Fig. 11 presents the equilibrium sex ratio for several values of c and the full range of possible selfing values. For *Plasmodium*, when $c = 8$ (the maximum suspected for these parasites), a single-clone infection should produce 11% males, with a greater number of males as the number of clones (adjusted for their relative abundance) increases.

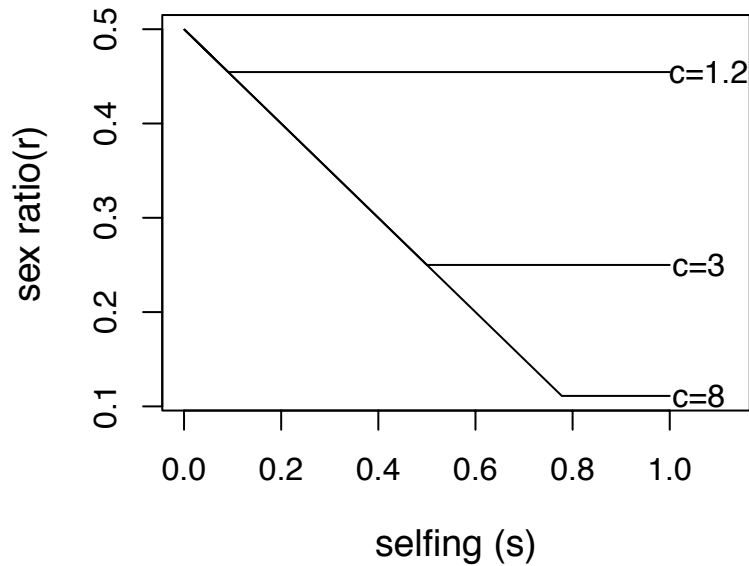


Figure 11: Predicted relationship between selfing (s) and sex ratio (r). For high selfing rates, the sex ratios predicted are often limited by the fecundity of males (c). The three values of c shown here are the maximum male fecundity of *Plasmodium* gametocytes ($c = 8$), the median male fecundity of *P. mexicanum* reported by Neal (2011; $c = 3$), and the median male fecundity of *P. mexicanum* if only 40% of gametes are successful at mating ($c = 1.2$). Expressions for the relationships shown are given in the text.

The LMC model assumes that all offspring in a patch are able to mate with one another, but West et al. (2001, 2002) recognized that factors such as very low gametocyte density, a strong anti-gametocyte immune response, or a quickly clotting blood meal

could limit the number of gametes able to interact with one another within a patch, creating extremely local mating groups. Small breeding groups and a strongly female-biased sex ratio combined make it likely that there will not be enough males present to mate all females. Under such conditions, more males should be produced to ensure union with female gametes, an extension of LMC referred to as Fertility Insurance. This is particularly true if fecundity is also low, and the interaction of mating group size and male fecundity can further decrease the degree of female bias predicted (Gardner et al. 2003). In summary, the model predicts that three factors influence the sex ratios in malaria parasites: number of clones in infections and their relative proportions (LMC), male fecundity (LMC and Fertility Insurance), and number of gametocytes in a mating group (Fertility Insurance).

Tests of the LMC model have produced mixed results, with some studies showing a good fit (Read et al. 1995; Reece et al. 2008; Sowunmi et al. 2009; Neal 2011), and others failing to support the predictions of the model (Shutler et al. 1995; Osgood and Schall 2004). Indeed, studies reach contrary conclusions for almost every factor likely to influence gametocyte sex ratios. For example, gametocyte density (important for Fertility Insurance) can be negatively related to sex ratio (Robert et al. 2003; Merino et al. 2004; Reece et al. 2008), positively related (Pickering et al. 2000, Schall 2000), or not related (Neal and Schall 2010). The age of the infection, which likely influences the strength of the immune attack, can be related to increase in male gametocytes (Paul et al. 1999; Osgood et al. 2002; Robert et al. 2003; Paul et al. 2002), or not (Schall 1989; Osgood and Schall 2004; Neal and Schall 2010). The best support for the model comes from experimental infections of chicken malaria (Paul 2000) or rodent malaria in

laboratory mice (Reece et al. 2008). Studies on natural infections need a measure of both gametocyte sex ratio and potential selfing of clones. In most such studies, clonal diversity is not directly measured, but instead surrogates are used such as prevalence of the parasite in a host population (Read et al. 1995; Shutler et al. 1995), the number of donor infections combined in experimental infections (Osgood and Schall 2002), or parasitemia (assumed higher in multi-clone infections due to competition; Pickering et al. 2000, Schall 2000).

Summarizing this model, we make several predictions for our study of both natural and experimental infections of *P. mexicanum*. In making these predictions, we assume that *P. mexicanum* is able to facultatively adjust its sex ratio in response to conditions within the host. Our predictions are: first, gametocyte sex ratio for infections with a single genotype will be between 25% and 45% male based on two estimates of *P. mexicanum* male fecundity, which are lower than the maximum of 8. The modal number of exflagellating male gametes seen for *P. mexicanum* is 3 (Neal 2011), which gives a predicted sex ratio of 25% males ($1/1+3$). However, not all male *Plasmodium* gametes may mate (up to 60% of gametes may be malformed [Sinden 1983; Sinden et al. 1978]), and Neal (2011) found a good match between the sex ratios predicted by fecundity and those observed for individual single-clone infections if it was assumed that only 40% of gametes were successful, giving an effective male fecundity of 1.2. This estimate yields a sex ratio of 45% males ($1/1+1.2$). Second, gametocyte sex ratio should shift from female biased in single clone infections toward more males as clone number and diversity of clones (equal relative proportions) increases. Third, all else being constant, lower gametocyte density will favor a higher proportion of males.

Materials and Methods

Study site, sampling and natural infections

Naturally infected and not infected fence lizards were collected at the University of California Hopland Research and Extension Center and two private ranches near the town of Hopland in Mendocino County, California, USA (Schall 1996; Fricke et al. 2010). Lizards were captured using a noose on a fishing pole, and a few drops of blood were taken by toe clip to produce a blood smear for staining (Giemsa) and microscopic examination. Several drops were also dried and frozen for genetic analysis. Infected lizards were identified by scanning slides at 1000X for at least 3 minutes. The natural infections surveyed in this study were all sampled from 2007 – 2009.

Experimental manipulation of clonal diversity

The data on experimental infections reported in this paper are taken from two experiments in which infections were generated via intraperitoneal injection of blood from naturally infected donor lizards into not infected recipient lizards. Detailed methods are provided by Osgood and Schall (2004) and Vardo-Zalik and Schall (2009). The total number of asexual stage parasites injected into each lizard was approximately 2×10^5 for all treatments in both experiments, and both experiments included infections initiated with blood from individual donors (“single-donor infections”) and blood combined from multiple donors (“multiple-donor infections”). The first experiment (“Experiment I”, Osgood and Schall 2004) was conducted in May – September 2001 and had 15 total donors. Single-donor infections were initiated for each of the donors in 3 – 10 recipient

lizards per donor, and 5 groups of multi-donor infections were initiated, each combining blood from 3 donors in 10 – 18 recipient lizards. This study used 7 single-clone and 7 multi-clone donors. The second experiment (“Experiment II”, Vardo-Zalik and Schall 2009) was conducted June – August 2005 and had 5 total donors. Seven – 8 single donor infections were initiated from each of the first 4 donors, and 8 – 14 multiple donor infections were initiated with each of 4 combinations of 2 – 4 donors. Experiment II used 2 single-clone and 3-multi-clone donors.

Gametocyte sex ratios and gametocytemia

Gametocytemia was determined by counting the number of gametocytes in 1000 erythrocytes from microscope fields sampled over the entire blood smear. Gametocyte sex ratios were estimated for every infection by counting mature male and female gametocytes (with characteristic shape, size, and staining; Schall 1989) until at least 100 were scored or for one hour of scanning. Most counts reached 100 gametocytes, and none with < 50 gametocytes scored were included in the analysis (see results for sample sizes). Sex ratio counts for the experimental infections were all performed by ATN and the blood smears from which counts were taken were made approximately 11 weeks after the infections were initiated (the latest date available for Experiment II). Sex ratio counts for the 2007 and 2008 natural infections were performed by ATN, and those for 2009 by ATN and an assistant. Quality control was tested by both observers counting 6 smears with very similar results ($P > 0.05$, paired t-test). Counts from infections for which counts were performed by both observers are summed for analysis because counts were made from different parts of the smear.

Clone numbers and clonal diversity

DNA was extracted from dried, frozen blood dots made on the same day as the blood smears using the DNeasy kit (Qiagen, Valencia, CA). For the natural infections, we amplified the DNA using PCR at 4 microsatellite loci (Pmx306, Pmx732, Pmx747, and Pmx839) with the primers and conditions presented by Schall and Vardo (2007). For the experimental infections, each infection was genotyped for the 2 of these 4 loci that had the most alleles in the donor infection(s). Previous studies found that these markers, although themselves presumed neutral and noncoding, are associated with variation in sex ratio in single-clone infections (Neal and Schall 2010, 2013), and that single- and multi-clone infections (based on these markers) differ in other important life history traits, including rate of asexual replication and virulence measures (Vardo-Zalik and Schall 2009). These studies show that these microsatellites reveal relevant information about the genetic diversity of *P. mexicanum* infections.

After amplification, the product was run on an ABI Prism genetic analyzer (Cornell Core Labs, Ithaca NY) and electropherograms were viewed using GeneMapper 3.5 software and PeakScanner v1.0 software (both by Applied Biosystems). The software allowed visualization of the size of each fragment (length of microsatellite allele based on number of ATT repeats) as well as the strength of the fluorescent signal. The peak height (i.e. strength of fluorescence) on electropherograms corresponds to the relative abundance of the allele in the sample (method verified in Vardo-Zalik et al. 2009; Ford et al. 2010; Ford and Schall 2011). Because all stages of the malaria parasite in a vertebrate host are haploid, each allele at a locus indicates a separate clone.

In the published derivations of sex ratio predictions based on LMC, sex ratio is predicted based on either the number of equally abundant clones or the selfing rate. The minimum number of clones in an infection was estimated as the maximum number of alleles at any one locus. However, not all clones are equally abundant, so we needed a measure of selfing as a predictor of sex ratio. Because selfing depends not only on the relative proportions of clones within an infection but also on the sex ratios produced by each clone, we were not able to estimate it directly from our data. Instead, we used a diversity measure (Hurlbert's $PIE = 1 - \sum p_i^2$; p_i = [height of peak i]/[sum of all peak heights]; no bias correction factor because genetic analysis samples so many parasites that bias correction has almost no effect, Gotelli 2008) to identify infections that had high and low clonal diversity. Diversity takes into account both clone numbers and their relative abundances. With this measure, an infection with a single clone has a diversity of 0, and an infection in which each individual parasite was a different clone would have a diversity of 1. Infections with higher diversity therefore have more clones in more equal proportions, and should therefore have less female-biased sex ratios. We calculated clonal diversity for each locus independently and used the maximum diversity at any one locus in the analysis.

Analysis

Wilson and Hardy (2002) recommend analysis of sex ratio data by generalized linear models (GLM) with binomial error structure. However, the sex ratio data from all three data sets showed significant deviation from a binomial distribution (fit to rounded

sex ratio $\times 100$, $X^2 > 240$, $P < 0.0001$) but not from a normal distribution ($X^2 < 1$, $P > 0.13$). Additionally, for p close to 0.5, the binomial distribution is very similar to a normal distribution with mean np and variance $np(p - 1)$, and the average sex ratios observed for *P. mexicanum* are generally between 0.4 and 0.5 (Neal and Schall 2010; Neal 2011; Osgood and Schall 2004; Osgood et al. 2002; 2003; Schall 1989). Therefore, linear models were applied. All analysis on sex ratio was weighted by the number of mature gametocytes counted because we have more confidence in ratios with more gametocytes sampled.

The goodness of fit tests mentioned above were performed in JMP 9.0.0 (SAS Institute Inc.), and all remaining analysis was performed in R version 2.12.2 (R Development Core Team, Vienna, Austria). We used multiple regression analysis to examine the relationship of the variables expected to influence sex ratio on the sex ratios observed. These variables include number of clones, gametocytemia, and number of donors (1 vs. multiple). The analysis was performed separately for each data set (natural infections, Experiment I, Experiment II). Number of clones is not predicted to be linearly related to sex ratio, but the inverse of clone number is over the range where sex ratio is not constrained by male fecundity (see Fig. 11; $1/N = s$ for equally abundant, unrelated clones), so clone number was transformed to its inverse for use in the multiple regression analysis. Gametocytemia was cube root transformed prior to analysis to reduce the degree of skewness in the data. We therefore fit multiple regression models to sex ratio with all combinations of $1/\text{clone number}$, $\text{gametocytemia}^{1/3}$, $1/\text{clone number} * \text{gametocytemia}^{1/3}$, and number of donor infections (experimental infections only) as predictor variables and ranked the resulting models based on their F-statistic to determine

the best combination(s) of predictor variables (Gotelli and Ellison 2004). The LMC model predicts a specific direction for the relationship between clonal diversity and gametocyte sex ratio and between gametocyte density and sex ratio (positive for proportion of male gametocytes vs. clonal diversity and negative for gametocyte density), so one-tailed significance tests were reported when these variables were included in the best model.

To determine whether the clonal diversity of infections affected the sex ratio (and not just the absolute number of clones), we ranked the infections based on the clonal diversity measure and divided them into thirds. We compared the sex ratios of infections with the lowest third vs. highest third of clonal diversity using a t-test. The middle third of infections were excluded for two reasons: First, electropherogram peak heights are related to the relative abundance of alleles, but not with great precision (Ford et al. 2010 provide confidence intervals), so removing the middle third ensures that there are true differences in diversity between the groups being compared. Second, the effect of selfing on sex ratios for a *Plasmodium* species with low male fecundity should be obvious only for the highest diversity infections (Fig. 11, esp. $c = 1.2$).

Results

Natural Infections

Of 223 infected lizards sampled over three seasons, 194 were successfully genotyped to determine number of clones present and their relative proportions. We were unable to genotype the remaining 28 infections for at least one of the loci (and thus had incomplete data), so these infections were excluded from analysis. These were primarily

infections with very low parasitemia, and thus PCR amplification did not yield product that could be successfully run on the analyzer for one or more loci. Of the 194, we were able to obtain sex ratio counts of at least 50 mature gametocytes for 184 infections, and the remaining 10 infections were also excluded from analysis. Summary data on sample sizes, sex ratio, clone numbers, clonal diversity, and gametocytemia are presented in Table 4.

Table 4: Summary statistics for each of the three data sets examined in this paper. The three data sets are natural infections and experimentally induced infections initiated for 2 previous studies. Group indicates the year samples were taken for natural infections and the number of donors and clones for experimental infections (s/s = single-donor, single-clone infections; s/m = single-donor, multi-clone; m/m = multi-donor, multi-clone). The calculation of clone number and clonal diversity are explained in the methods section. Sex ratio estimates are recorded as the proportion of mature gametocytes counted that were male based on at least 50 mature gametocytes per infection. Gametocytes indicates the number of gametocytes (mature and immature) counted in a sample of 1000 erythrocytes. Measures are reported as mean (median, minimum – maximum).

Data Set	Group	N	Clones	Diversity	Sex Ratio	Gametocytes
Natural	2007	36	2.64 (2, 1-5)	0.44 (0.47, 0-0.74)	0.46 (0.47, 0.30-0.57)	14.7 (6, 1-148)
Natural	2008	53	1.76 (2, 1-4)	0.27 (0.31, 0-0.72)	0.41 (0.39, 0.25-0.58)	16.6 (8, 0-205)
Natural	2009	95	1.71 (2, 1-4)	0.21 (0.16, 0-0.64)	0.46 (0.46, 0.33-0.61)	16.8 (7, 0-169)
Natural	All	184	1.90 (2, 1-5)	0.27 (0.31, 0-0.74)	0.44 (0.45, 0.25-0.61)	16.3 (7, 0-205)
Study I	s/s	26	1 (1, 1-1)	0 (0, 0-0)	0.43 (0.44, 0.29-0.55)	29.0 (22.5, 2-117)
Study I	s/m	21	2.10 (2, 2-3)	0.48 (0.48, 0.35-0.64)	0.44 (0.48, 0.35-0.57)	33.6 (23, 5-112)
Study I	m/m	30	4.40 (4, 3-5)	0.60 (0.66, 0.13-0.76)	0.48 (0.48, 0.39-0.56)	35.0 (33.5, 1-91)
Study I	all	77	2.62 (2, 1-5)	0.36 (0.46, 0-0.76)	0.45 (0.46, 0.29-0.57)	32.6 (26, 1-117)
Study II	s/s	15	1 (1, 1-1)	0 (0, 0-0)	0.39 (0.39, 0.30-0.52)	13.3 (9, 1-47)
Study II	s/m	11	2 (2, 2-2)	0.32 (0.46, 0.10-0.49)	0.41 (0.41, 0.32-0.49)	23.2 (16, 1-78)
Study II	m/m	37	4.1 (4, 2-6)	0.53 (0.56, 0.13-0.77)	0.40 (0.41, 0.22-0.53)	23.8 (12, 0-156)
Study II	all	63	2.98 (2, 1-6)	0.37 (0.46, 0-0.77)	0.40 (0.40, 0.22-0.53)	21.2 (12, 0-156)

Sex ratios ranged from 0.25 – 0.61 proportion males (median 0.45, mean 0.44, Table 4, Fig. 12A). The sex ratio of single-clone infections differed significantly from the value of 0.25 predicted for a male gametocyte fecundity of $c = 3$ but not from the 0.45 predicted if $c = 1.2$ (mean = 0.429, 95% CI: 0.419 – 0.459). The multiple regression model with the highest F included only transformed gametocytemia ($F = 3.75$, $P_{\text{model}} = 0.054$), though infections with higher gametocytemia tended to have less female-biased sex ratio, contrary to the predictions of Fertility Insurance ($\beta_1 = 0.010$, $t = 1.937$, $P_{t < 0} = 0.973$). Gametocytemia was negatively correlated with clonal diversity, contrary to prediction ($t = -2.026$, $P = 0.0442$, regression). Analysis with clonal diversity confirmed the lack of significant relationship between clones and sex ratio (Fig. 13a – b; mean sex ratios: high diversity = 0.436, low diversity = 0.439; $t = -0.203$, $P_{t > 0} = 0.580$; diversity cutoffs: high > 0.448 , low = 0). In summary, the factors predicted to be important for gametocyte sex ratio- clone number, clonal diversity, and gametocyte density- did not explain the variation in sex ratio for natural infections.

Experiment I

Of 143 infections initiated for this experiment, blood smears and dots made 11 weeks post inoculation were available for 86 infections. Of these, we were unable to either successfully genotype or obtain sex ratio counts with at least 50 gametocytes for 9 infections, bringing the total number of infections included in the analysis to 77. Summary data on these 77 infections is provided in Table 4.

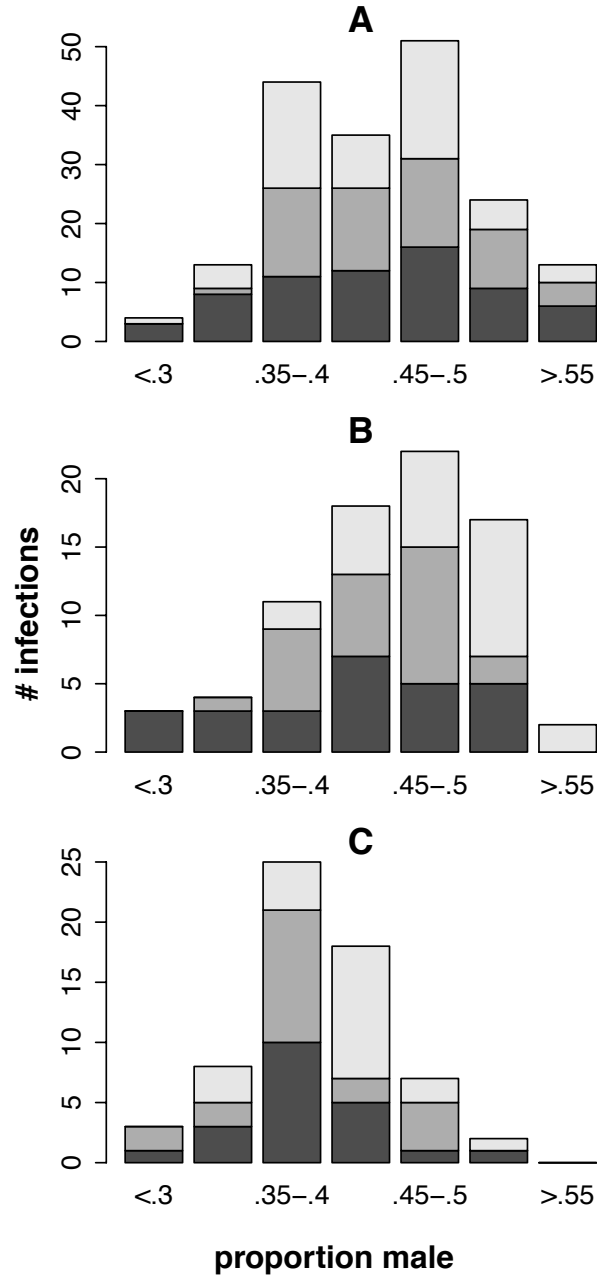


Figure 12: Distribution of sex ratios observed in natural (A) and experimental (B- Experiment I, C- Experiment II) infections of the lizard malaria parasite *Plasmodium mexicanum*. Shading indicates the relative clonal diversity of the infections: dark gray is low clonal diversity, medium gray is medium clonal diversity, and light gray is high clonal diversity.

Sex ratios ranged from 0.29 to 0.57 proportion male (mean = 0.45, median = 0.46,

Fig 12B). Sex ratios of single clone infections were significantly different from 0.25

predicted for $c = 3$ but not from 0.45 for $c = 1.2$ (mean = 0.428, 95% CI: 0.418 – 0.457).

The 2 multiple regression models with the highest F statistics each contained a single predictor variable: number of clones ($F = 11.34$) and number of donors (single vs. multiple, $F = 10.93$). Both models showed a relationship in the direction predicted by LMC: there was a negative relationship between the inverse clone number and sex ratio ($t = -3.368$, $P_{t<0} = 0.006$), and a positive relationship between donor number and sex ratio (i.e. multi-donor infections had higher sex ratios, $t = 3.306$, $P_{t>0} = 0.0007$). All other models had $F < 6.5$. Comparing infections with high vs. low clonal diversity confirmed this pattern: high diversity infections (diversity > 0.528) had significantly higher sex ratios than low diversity infections (Fig. 13c – d; diversity = 0; mean sex ratios: high = 0.49, low = 0.43; $t = 3.56$, $P_{t>0} = 0.0004$). In contrast to a prediction of Fertility Insurance, sex ratio and gametocytemia were not correlated, nor was gametocytemia significantly correlated with clonal diversity ($t = 0.738$, $P = 0.463$, regression).

Experiment II

Sixty-three of the 69 infections initiated for this experiment were successfully genotyped and 50 mature gametocytes counted for samples taken 11 weeks after the infections were initiated. Table 4 provides summary data.

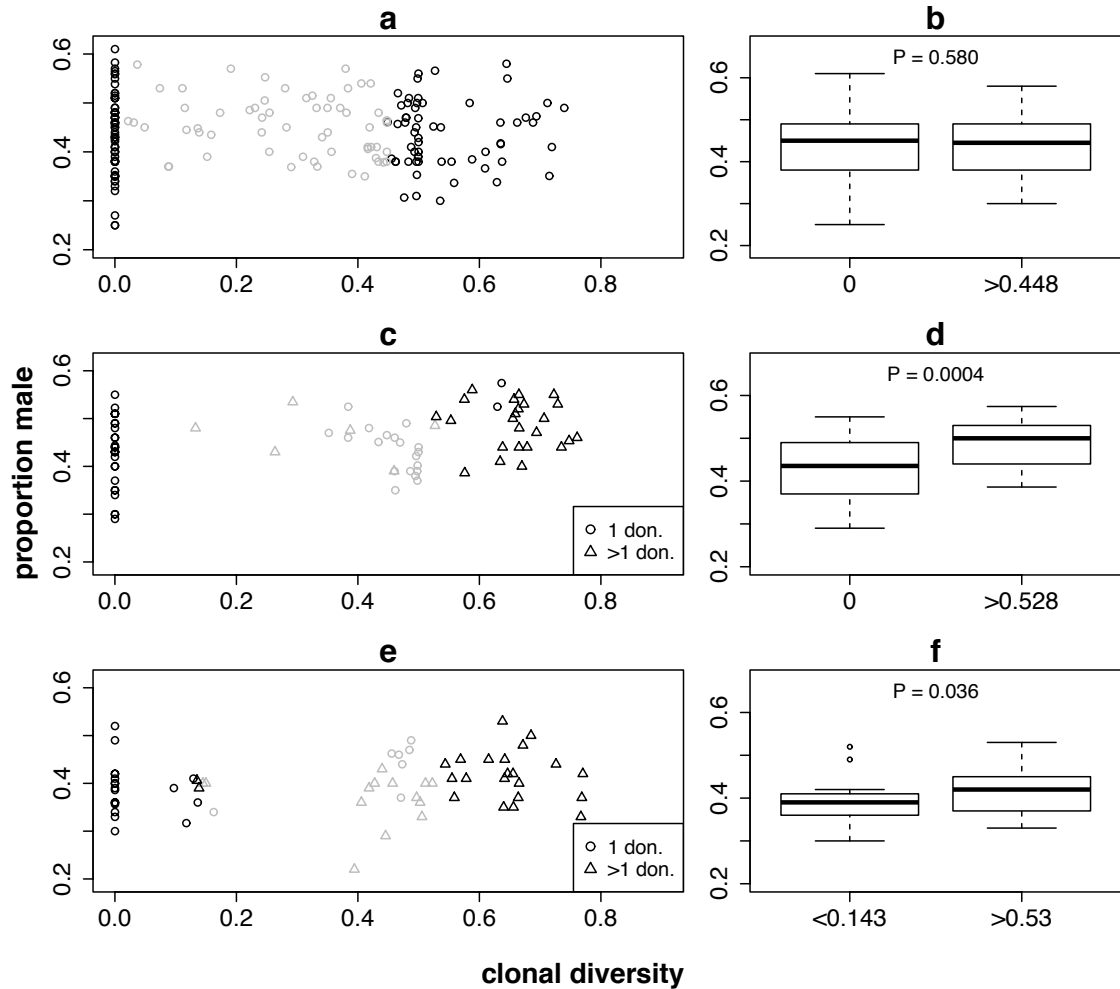


Figure 13: Relationship between clonal diversity and sex ratio in natural and experimental infections of *Plasmodium mexicanum*. The first column of graphs shows individual data points. The shape of the point indicates number of donors (experimental infections only; see legend). Black points indicate infections with the highest and lowest 1/3 of clonal diversity measures; gray points are the middle third and were excluded from the diversity analysis. The second column of graphs shows a comparison of the sex ratios of infections with high (highest 1/3) and low (lowest 1/3) clonal diversity. (a) and (b) are data from natural infections collected over 3 years. (c) and (d) are data from experimental infections from Experiment I (e) and (f) are data from experimental infections from Experiment II.

Sex ratios ranged from 0.22 to 0.53 proportion male (mean/median = 0.40, Fig 12C). Single clone infections had sex ratios significantly higher than 0.25 and lower than 0.45 (mean = 0.392, 95% CI: 0.360 – 0.424). The multiple regression model with the highest F value contained only gametocytemia as a predictor variable ($F = 3.09$, $P_{\text{model}} = 0.084$), but the relationship was positive, not negative as predicted by Fertility Insurance

($\beta_1 = 0.01$, $t = 1.76$, $P_{t<0} = 0.958$). Gametocytemia is not significantly associated with clonal diversity ($t = 0.512$, $P = 0.611$, regression). For all other models, F was less than 1.7. Clonal diversity was significantly related to gametocyte sex ratio with higher sex ratios seen in more clonally diverse infections (Fig. 13e – f; mean sex ratios: high = 0.42, low = 0.39; $t = 1.85$, $P_{t>0} = 0.036$; diversity cutoffs: high > 0.53, low < 0.143).

Comparison of natural vs. experimental infections

Natural infections had a lower clonal diversity than the experimental infections (medians: 0.30 natural, 0.46 experimental, $P = 0.0005$, Kruskal-Wallis), though the trend was opposite if multi-donor infections were excluded (medians: 0.30 natural, 0.46 experimental, $P = 0.007$). Gametocytemia was higher in experimental infections whether or not multiple-donor infections were included (medians: 7 natural, 17 experimental, $P < 0.0001$). Sex ratio tended to be lower in experimental infections (means: 0.443 natural, 0.428 experimental, $P = 0.057$), especially if multi-donor infections were excluded (means: 0.443 natural, 0.422 experimental, $P = 0.025$).

Discussion

Ronald Ross, one of the discoverers of the *Plasmodium* life cycle, witnessed the toll the parasite exacted from human populations, and balefully referred to malaria as the “million murdering death.” Today, a century later, hundreds of millions of people still are infected each year, with perhaps a million dying (Murray et al. 2012). Understanding the factors shaping gametocyte sex ratios should cast light on aspects of the parasite’s life history of public health significance, including clonal diversity, how those clones interact

within an infected host, gametocyte production, sexual recombination, and ultimate transmission success. Thus, this is a striking case of sex ratio theory having significance for human health and economic welfare. To date, though, few studies have explicitly tested the predictions of LMC and Fertility Insurance for individual infections using direct measures of the relevant variables, and none for a natural parasite-host system that has not been altered by intense public health measures such as drug treatment. This study therefore offers a valuable complement to the experimental studies on a laboratory model system (rodent malaria in laboratory mice), experiments for natural lizard and chicken malaria, as well as broad comparisons for natural malaria systems. We present results for a large sample of natural infections of a malaria parasite of lizards over a three year period and two experimental studies that manipulated the number of clones within infected lizards. We had available data on the relevant biological features needed to test the theory: reliable counts of gametocytes, number of clones, their relative abundance, male gametocyte fecundity, and gametocyte density. The results match the prediction of greater production of males with a lower chance of selfing for the experimental infections, but not for the natural infections. Gametocyte sex ratio was consistent with the low observed male fecundity for single-clone infections. However, sex ratio did not show the negative association with gametocytemia predicted by Fertility insurance, and in fact showed the opposite tendency in two of the three studies.

Local Mate Competition

LMC predicts that gametocyte sex ratios will be less female-biased when the probability of mating with closely related individuals is lower (Hamilton 1967). This

shift in sex ratio could result through local adaptation, i.e. all parasites in an area may produce a sex ratio that is adaptive based on the prevailing degree of clonal diversity (and other factors, such as those implicated in Fertility Insurance), or via phenotypic plasticity, i.e. parasites in individual infections may be able to detect relevant cues (presence/abundance of co-infecting clones, etc.) and shift their sex ratio accordingly. Here, we focused on the latter strategy: our goal was to determine whether *P. mexicanum* is able to facultatively shift its sex ratio in response to the number and relative abundance of co-infecting clones, which we measured using 4 microsatellite loci. Two testable predictions emerge from theory. First, in single-clone infections, a specific sex ratio is predicted based on the number of gametes produced by the male gametocyte. Male fecundity for *P. mexicanum* has been estimated as 1.2 - 3, so the predicted sex ratio in single-clone infections should be 0.25 – 0.45 proportion male. Second, the overall trend should be a decrease in female bias as clonal diversity increases (increase in number of clones and/or more equal relative proportions).

The sex ratio for single-clone infections for both natural and experimental infections was only slightly female biased. A measure of male fecundity based on number of gametes produced per male gametocyte ($c = 3$) predicts a significantly more female-biased sex ratio than observed (means for the three studies: 0.440, 0.428, 0.392). However, if developmental success of gametes is about 40% as determined for *P. falciparum* (Sinden 1983) and indirectly estimated for *P. mexicanum* (Neal 2011), male fecundity would be 1.2, and this predicts a sex ratio of 45% males, close to the observed value for all three studies.

A male fecundity as low as 1.2 would make it difficult to observe an effect of increasing clonal diversity on sex ratio, necessitating quite high clonal diversity for any shift in sex ratio to be predicted (Fig. 11). Data for natural infections of *P. mexicanum* showed no effect of the number of clones or clonal diversity on sex ratio. In contrast, results from two experimental studies showed a significant effect of clonal diversity on gametocyte sex ratio in the direction predicted by the LMC model. The experimental infections were generated by two researchers with somewhat different experimental designs conducted several years apart, and despite the only slight female-bias seen in infections of *P. mexicanum* (medians about 0.45 males), the overall trend supporting the model appeared for both experimental studies. What accounts for the conflict between natural and experimental infections? Three possible differences between natural and experimental infections could drive the disparate results.

First, the low male fecundity estimated for *P. mexicanum* would require a high clonal diversity, and thus low selfing, for a shift in gametocyte sex ratios to be predicted. The natural infections had a lower clonal diversity (median 0.31) than the experimental infections (median 0.46), which may have obscured effects predicted by LMC.

Second, in the experimental infections, all clones entered a host simultaneously, and we have a complete record of the history of the infection. Natural infections were gathered over the entire course of the warm season over three years, and thus clones may have been entering and leaving, which would be undetectable with only a single sample taken. Also, the microsatellite markers do not differentiate between asexual parasite stages (the replication stages) and the gametocytes. If clones are at different developmental stages, the relative proportions of all parasites and only gametocytes may

differ, causing our measure of clonal diversity not to reflect the true genetic diversity of the mating population.

Third, experimental infections may create combinations of clones in the vertebrate host that do not exist naturally. *Plasmodium mexicanum* shows signs of genetic differentiation at sites only 0.5 km distant (Fricke et al. 2010), and the donors used to initiate the experimental infections were often collected from different sites. Clones naturally found together may often be found together (because they readily transmit together to the insect host [Vardo-Zalik 2009]), and could cooperate to reach the overall infection sex ratio (all clones producing a female-biased sex ratio should increase zygote production and thus transmission success) or have a higher degree of relatedness than microsatellites would suggest. Thus, combining clones from different sites in experimental infections may increase the likelihood that clones are more diverse, increasing the likelihood of detecting a pattern.

Fertility Insurance

In addition to testing the predictions of LMC, this study tested one prediction of Fertility Insurance for *P. mexicanum*. Fertility Insurance predicts that factors reducing the probability of male gametes encountering and combining with female gametes should lead to an increased investment in males (up to a 1:1 sex ratio). These factors include low gametocyte density, host anemia, small vector blood meal size, a quickly clotting blood meal, and a strong immune response. We tested if gametocyte sex ratio was more female-biased in infections with higher gametocyte density. In 2 of the 3 studies, gametocytemia was a predictor of sex ratio in the multiple regression model, but in both

studies the trend was the opposite of that predicted by Fertility Insurance: both showed a positive rather than negative trend. *Plasmodium mexicanum* in general has high gametocytemia relative to malaria parasites for which a negative association between gametocytemia and sex ratio has been observed previously (Bromwich and Schall 1986; Taylor and Read 1997; Reece et al. 2008 [*P. chabaudi*]; Robert et al. 2003 and Sowunmi et al. 2009 [*P. falciparum*]), so it is quite possible that the lowest gametocytemias for *P. mexicanum* are not low enough to reduce fertilization rates and thus favor a higher production of males.

Positive associations between gametocytemia and sex ratio have been observed previously for *P. tropiduri* (Pickering et al. 2000) and *P. mexicanum* (Schall 2000). Pickering et al. (2000) attributed this positive association to infections with more clones likely having both higher gametocytemia and less biased sex ratios, though they did not measure clone numbers. This does not appear to be the case here, as gametocytemia is not positively associated with clonal diversity for any of the data sets.

While our results do not suggest that *P. mexicanum* has low enough gametocyte density to require production of additional males, other factors requiring sex ratio adjustment for Fertility Insurance that we did not measure directly may affect sex ratio evolution for this species. One example is rapid clotting of blood within the insect host. Although millions of erythrocytes may be taken in a blood meal, the meal may be rapidly divided into very small breeding groups if the blood quickly forms clots. Anecdotal evidence suggests that lizard blood clots more quickly than blood from mammals or birds (Neal and Schall 2010), as does blood taken from infected lizards relative to not infected animals (Vardo-Zalik and Schall 2008). Another example is vector size: sand flies, the

vectors of *P. mexicanum*, take smaller blood meals and thus consume fewer parasites than the mosquitoes and black flies that transmit mammalian and some avian malaria parasites (Shutler and Read 1998). Blood clotting and vector size were not considered in detail here because they are more likely to explain variation among species than within species, but they may well account in part for the only slight female bias often observed for *P. mexicanum*.

Additionally, anemia and strength of immune response are two factors that may affect selection on sex ratios within a species. While neither anemia nor immunity have been investigated in detail in relation to sex ratio for *P. mexicanum*, both have been associated with increasing sex ratio over time in other species (e.g. *P. gallinaceum* [Paul et al. 1999]; *P. gallinaceum* and *P. vinckei* [Paul 2000]), and *P. mexicanum* infections followed over time show no consistent shift in sex ratio (Schall 1989; Osgood and Schall 2004; Neal and Schall 2010). Also, a previous study attempted to down regulate the lizard host's immune system using testosterone, but no affect on sex ratio was observed (Osgood et al. 2003). Fertility Insurance does not appear to explain variation among infections in *P. mexicanum*, but may play a role in this species' relatively high sex ratios compared with other malaria parasites.

Conclusions

Data on gametocyte sex ratio for malaria parasites are still sparse, with less than a dozen species surveyed (reviewed in Schall 2009). Results show, however, that malaria parasites display a great diversity of sex ratios ranging from strongly female-biased to close to 1:1, with even a few reports of male-biased sex ratios.

Previous laboratory studies of both a rodent malaria parasite in a model host and a chicken malaria parasite found evidence of facultative shifts in sex ratio in response to environmental factors relevant to LMC and Fertility Insurance. Our study examined a natural *Plasmodium* system (the parasite in its natural vertebrate host), both in natural infections and infections with experimentally manipulated clonal diversity. Again, results concord broadly with the LMC model. The best studied *Plasmodium* species- *P. falciparum* of humans, *P. chabaudi* of rodents, *P. gallinaceum* of chickens, and *P. mexicanum* of lizards- are not closely related (Martinsen et al. 2008). Thus, overall, these studies argue that malaria parasites are able to assess the functional male gametocyte fecundity and detect the diversity of genetic clones within the infection to alter sex ratio as expected by the LMC model.

Our study of a natural *Plasmodium* system highlights several important issues. First, the male gametocyte fecundity is a life history trait that is central for an understanding of the variation in sex ratio. This is because low male fecundity can limit the range of sex ratio values over which an effect on sex ratio is predicted. Paul et al. (1999) suspected the importance of fecundity when following increases over time in the gametocyte sex ratio of *P. gallinaceum* in chickens, as expected if the functional male fecundity is reduced by action of the host immune system. Fertility Insurance also highlights the importance of any reduction in functional male fecundity (Gardner et al. 2003). Second, the clonal diversity within infections may be low for some species, even when parasite prevalence is high. Read et al. (1995) and Shutler et al. (1995) used prevalence as a proxy for clonal diversity within infections, but prevalence and clonal diversity may not be strongly related (Vardo and Schall

2007). Last, the Fertility Insurance addition to the LMC model notes that low gametocyte density would favor a less female biased sex ratio. This would be critical for species that produce few gametocytes in the vertebrate host blood (*P. falciparum*, for example [Taylor and Read 1997]), but not for other species that show high density of gametocytes such as we found for *P. mexicanum*.

When the LMC model was first applied to malaria parasites, Read et al. (1992) noted that traits related to sex ratio evolution, such as the interaction of parasite clones within the host and ultimate transmission success, could also be important for public health efforts to control one of the most important infectious diseases of human populations. Sex ratio theory has a central place in evolutionary biology, and its application to malaria parasites demonstrates both its generality (for even protists) and significance for applied biology as well as evolutionary theory.

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Literature Cited

- Alano, P. and R. Carter. 1990. Sexual differentiation in malaria parasites. *Ann. Rev. Microbiol.* 44: 429-449.
- Bromwich, C. R. and J. J. Schall. 1986. Infection dynamics of *Plasmodium mexicanum*, a malaria parasite of lizards. *Ecology* 67: 1227-1235.
- Charnov, E. L. 1982. The theory of sex allocation. Princeton Univ. Press, Princeton, NJ.
- Darwin, C. 1871. The descent of man, and selection in relation to sex. John Murray, London, U.K.
- Düsing, C. 1884. Die regulierung des geschlechtsverhältnisses bei der vermehrung der menschen, tiere und pflanzen. Fischer, Jena.
- Dye, C. and H. C. F. Godfray. 1993. On sex ratio and inbreeding in malaria parasite populations. *J. Theor. Biol.* 161: 131-134.
- Edwards, A. W. F. 2000. Carl Düsing (1884) on the regulation of the sex-ratio. *Theor. Popul. Biol.* 58: 255-257.
- Ford, A. F. and J. J. Schall. 2011. Relative clonal proportions over time in mixed-genotype infections of the lizard malaria parasite *P. mexicanum*. *Int. J. Parasitol.* 41: 731-738.
- Ford, A. F., A. M. Vardo-Zalik, and J. J. Schall. 2010. Relative clonal density of malaria parasites in mixed-genotype infections: validation of a technique using microsatellite markers for *Plasmodium falciparum* and *P. mexicanum*. *J. Parasitol.* 96: 908-913.
- Frank, S. A. 1986. Hierarchical selection theory and sex ratios. I. General solutions for structured populations. *Theor. Popul. Biol.* 29: 312-342.

- Fricke, J. M., A. M. Vardo-Zalik, and J. J. Schall. 2010. Geographic genetic differentiation of a malaria parasite, *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*. *J. Parasitol.* 96: 308-313.
- Gardner, A., S. Reece and S. West. 2003. Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *J. Theor. Biol.* 223: 515-521.
- Garnham, P. C. C. 1966. Malaria parasites and other haemosporidians. Blackwell, Oxford.
- Ghiselin, M. T. 1974. The economy of nature and the evolution of sex. University of California Press, Berkeley.
- Gotelli, N. J. 2008. A primer of ecology. 4th ed. Sinauer Associates, Sunderland, MA.
- Gotelli, N. J. and A. M. Ellison. 2004. A primer of ecological statistics. 1st ed. Sinauer Associates, Sunderland, MA.
- Hamilton, W. D. 1967. Extraordinary sex ratios. *Science* 156: 477-488.
- Lobo, C. A. and N. Kumar. 1998. Sexual differentiation and development in the malaria parasite. *Parasitol. Today* 14: 146-150.
- Martinsen, E. M., S. L. Perkins, and J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molec. Phylogenet. Evo.* 47: 261-273.
- Merino, S., G. Tomás, J. Moreno, J. J. Sanz, E. Arriero, and C. Folgueira. 2004. Changes in *Haemoproteus* sex ratios: fertility insurance or differential sex lifespan? *Proc. R. Soc. Lond. B* 271: 1605-1609.
- Murray, C. J. L., R. C. Rosenfeld, S. S. Lim, K. G. Andrews, K. J. Foreman, D. Haring, N. Fullman, M. Naghavi, R. Lozano and A. D. Lopez. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet* 379: 413-431.
- Neal, A. T. 2011. Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 138: 1203-1210.
- Neal, A. T. and J. J. Schall. 2010. Gametocyte sex ratio in single-clone infections of the malaria parasite *Plasmodium mexicanum*. *Parasitology* 137: 1851-1859.
- Neal, A. T. and J. J. Schall. 2013. Life history focus on a malaria parasite: linked traits and variation among genetic clones. *Evol. Ecol.* (in press)

- Osgood, S. M., R. J. Eisen and J. J. Schall. 2002. Gametocyte sex ratio of a malaria parasite: experimental test of heritability. *J. Parasitol.* 88: 494-498.
- Osgood, S. M. R. J. Eisen, A. R. Wargo and J. J. Schall. 2003. Manipulation of the vertebrate host's testosterone does not affect gametocyte sex ratios of a malaria parasite. *J. Parasitol.* 89: 190-192.
- Osgood, S. M. and J. J. Schall. 2004. Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* 128: 23-29.
- Paul, R. E. L. 2000. Sex determination in malaria parasites. *Science* 287: 5450.
- Paul, R. E. L. and K. P. Day. 1998. Mating patterns of *Plasmodium falciparum*. *Parasitol. Today*: 14: 197-202.
- Paul, R. E. L., A. Raibaud and P. T. Brey. 1999. Sex ratio adjustment in *Plasmodium gallinaceum*. *Parassitologia* 41: 153-158.
- Paul, R. E. L., P. T. Brey and V. Robert. 2002. *Plasmodium* sex determination and transmission to mosquitoes. *Trends Parasitol.* 18: 32-38.
- Pickering, J., A. F. Read, S. Guerrero, and S. A. West. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evol. Ecol. Res.* 2: 171-184.
- Read, A. F., M. Anwar, D. Shutler, and S. Nee. 1995. Sex allocation and populations structure in malaria and related parasitic protozoa. *P. Roy. Soc. Lond. B. Bio.* 260: 359-363.
- Read, A. F. and K. P. Day. 1992. The genetic structure of malaria parasite populations. *Parasitol. Today* 8: 239-242.
- Read, A. F., A. Narara, S. Nee, A. K. Keymer, and K. P. Day. 1992. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Reece, S. E., D. R. Drew and A. Gardner. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614.
- Robert, V., C. S. Sokhna, C. Rogier, F. Ariey, and J.-F. Trape. 2003. Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* 127: 1-8.
- Schall, J. J. 1989. The sex ratio of *Plasmodium* gametocytes. *Parasitology* 98: 343-350.
- Schall, J. J. 1996. Malarial parasites of lizards: diversity and ecology. *Adv. Parasit.* 37: 255-333.

- Schall, J. J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.
- Schall, J. J. 2009. Do malaria parasites follow the algebra of sex ratio theory? *Trends Parasitol.* 25: 120-123.
- Schall, J. J., A. Bennett and R. Putnam. 1982. Lizards infected with malaria: physiological and behavioral consequences. *Science* 217: 1057–1058.
- Schall, J. J. and A. M. Vardo. 2007. Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Mol. Ecol. Notes* 7: 227-229.
- Shutler, D., G. F. Bennett, and A. Mullie. 1995. Sex proportions of *Haemoproteus* blood parasites and local mate competition. *P. Natl. Acad. Sci. USA* 92: 6748-6752.
- Sinden, R. E. 1983. The cell biology of sexual development in *Plasmodium*. *Parasitology* 86: 7-28.
- Sinden, R. E., E. Canning, R. Bray and M. Smalley. 1978. Gametocyte and gamete development in *Plasmodium falciparum*. *Proc. R. Soc. Lond. B* 201: 375-399.
- Sowunmi, A., G. O. Gbotosho, C. T. Happi, O. A. Folarin, and S. T. Balogun. 2009. Population structure of *Plasmodium falciparum* gametocytes in malarious children in an endemic area. *Parasitol. Int.* 58: 438-443.
- Taylor, L. H. and A. F. Read. 1997. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today* 13: 135-140.
- Taylor, P. D. and M. G. Bulmer. 1980. Local mate competition and the sex ratio. *J. Theor. Biol.* 86: 409-419.
- Vardo, A. M. and J. J. Schall. 2007. Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Mol. Ecol.* 16: 2712-2720.
- Vardo-Zalik, A. M. 2009. Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host. *Int. J. Parasitol.* 39: 1573-1579.
- Vardo-Zalik, A. M., A. F. Ford, and J. J. Schall. 2009. Detecting number of clones, and their relative abundance, of a malaria parasite (*Plasmodium mexicanum*) infecting its vertebrate host. *Parasitol. Res.* 105: 209-215.

Vardo-Zalik, A. M. and J. J. Schall. 2008. Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 135: 1363-1372.

Vardo-Zalik, A. M. and J. J. Schall. 2009. Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* 90: 529-536.

West, S. 2009. Sex allocation. Princeton University Press, Princeton.

West, S. A., S. E. Reece, and A. F. Read. 2001. Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends Parasitol.* 17: 525-531.

West, S. A., T. G. Smith, S. Nee and A. F. Read. 2002. Fertility insurance and the sex ratios of malaria and related Hemospororin blood parasites. *J. Parasitol.* 88: 258-263.

Wilson, D. S. and R. K. Colwell. 1981. Evolution of sex ratio in structured demes. *Evolution* 35: 882-897.

Wilson, K. and I. C. W. Hardy. 2002. Statistical analysis of sex ratios: an introduction. In: Sex ratios: concepts and research methods. I. C. W. Hardy ed. Cambridge Univ. Press, Cambridge, U.K.

CHAPTER 5: Malaria parasite sex ratio and clonal diversity at sites with long-term differences in the proportion of hosts infected

Allison T. Neal and Annika T. Nilsson

Abstract

A model from sex ratio theory called local mate competition (LMC) predicts a positive relationship between the number of genetic lineages of a sexually dioecious organism in a habitat patch and the proportion of males in that patch. This model can be tested for malaria parasites by comparing the proportion of hosts in a population that are infected (parasite prevalence) with the average sex ratio of those infections. However, such tests assume that the number of parasite lineages can be predicted from parasite prevalence because lineages will be distributed among hosts following the negative binomial distribution. If these assumptions hold, populations with higher parasite prevalence should have more lineages per host and a higher proportion of male parasites. Here, we test these predictions for a malaria parasite *Plasmodium mexicanum* in its host, the western fence lizard *Sceloporus occidentalis*. We also test whether the distribution of parasite lineages is consistent with the negative binomial distribution. Samples were collected at field sites with chronic high (20 – 27% infected overall) and low (1 – 10%) prevalence of the parasite over 30 years. The number of lineages per host was assessed using microsatellite loci and sex ratios were estimated by microscopic examination. We used multiple summary statistics to compare lineage numbers and genetic diversity between high and low prevalence sites, but none showed a significant difference. Sex ratios also did not differ between high and low prevalence sites. Additionally, the distribution of clones among infections deviated significantly from a negative binomial

distribution and suggested a positive association among sites between mean lineages per infection and the degree of clonal aggregation. Collectively, these results suggest that parasite prevalence may not be a reliable correlate of lineage number.

Introduction

Darwinian medicine seeks to incorporate evolutionary theory into medical practice and public health, with goals such as improving delivery of therapy, preventing drug resistance, and even manipulating pathogens or vectors to reduce transmission and virulence (Nesse 2007, Read et al. 2009, Read et al. 2011). One promising example is the application of sex ratio theory, a successful application of evolutionary biology, toward an understanding of clonal diversity and inbreeding rates for malaria parasites (Read et al. 1992; Neal and Schall 2014). The genetic diversity of the parasite within its vertebrate host, and how genotypes recombine in the insect vector, has ties to medically important traits such as virulence and the spread of drug resistance (Babiker and Walliker 1997, Walliker et al. 1998, Read and Taylor 2001). Under certain conditions characteristic of malaria parasites (see below), the theory predicts that sex ratios will vary with the degree of selfing (defined here as mating between gametes derived from the same genetic lineage) or inbreeding (which is mathematically equivalent to selfing in this context; Dye and Godfray 1993) in a specific, quantitative pattern. Some authors have argued that estimating inbreeding rates from sex ratios may even be preferable to using genetics due to its simplicity and lower cost (important for field conditions) and evolutionarily relevant scaling (i.e. rather than arbitrarily being measured at, for example, the village or regional level; West 2009, Nee et al. 2002). To be employed with confidence, however, the method must be validated, and tests of the predicted

relationship between sex ratio and selfing have produced mixed results (in support: Read et al. 1992 with inbreeding estimate from Paul et al. 1995, Read et al. 1995, Reece et al. 2008, Neal and Schall 2014, Sowunmi et al. 2009; failure to support: Read et al. 1992 with inbreeding estimate from Anderson et al. 2000, Shutler et al. 1995, Osgood and Schall 2004). One of the difficulties for testing these theoretical predictions is the lack of established molecular genetic methods in many parasite-host systems. This has led to creative stand-ins for selfing (reviewed by Schall 2009), but the validity of these alternatives is largely untested and may itself contribute to the production of conflicting results. Here we focus specifically on the utility of the prevalence of malaria parasites (proportion of hosts infected) as an estimator of parasite selfing rates and sex ratios.

Malaria parasites (*Plasmodium* and related genera; Martinsen et al. 2008) reproduce asexually in a vertebrate host (bird, squamate reptile, or mammal), eventually producing sexually dimorphic cells called gametocytes, which are the only stage that survive transmission to the insect vector. Due to the asexual replication that precedes gametocyte production, many of these cells are genetically identical, with most infections containing only one or a few clonal lineages of parasite (Read and Day 1992, Paul and Day 1998). Male and female gametocytes form gametes and mate in the insect vector, which generally have consumed blood from a single infected host (Walliker et al. 1998). This population structure, with small groups of highly related individuals separated from other such groups during mating by the patchy distribution of suitable habitat (in this case, insect vectors), ensures that much of the mating, and competition among males for mates, occurs between closely related individuals and accords well with a model from sex ratio theory called Local Mate Competition (LMC; Read et al. 1992, Hamilton 1967).

This model predicts female-biased sex ratios, with the degree of bias dependent on the number of lineages present in a patch (more biased sex ratios are predicted when there are fewer lineages; Hamilton 1967, Taylor and Bulmer 1980). The LMC model thus predicts that gametocyte sex ratios will be strongly female biased when only a single clone of parasites is present, but tend toward more males when the parasite pool is genetically complex.

Testing this prediction requires a way of evaluating the clonal diversity of infections, but most non-human malaria parasites lack well-characterized, variable genetic markers for direct estimation of the number of genotypes within the vertebrate host. Read et al. (1995) proposed that the parasite prevalence could instead be used to estimate the average number of clones per infection. They derived an expression that links prevalence and sex ratio based on the assumption that clonal diversity among hosts follows a negative binomial distribution. The predicted sex ratio (r^* = proportion of male gametocytes) was derived as $r^* = (1/2) [1 - \{P/[k(1/P - 1) - k]\}]$, where P is the proportion of hosts infected at a site, and k is the dispersion parameter, which is inversely related to the degree of parasite aggregation within hosts (Read et al. 1995 use values of k from 0.5 – 2). Low values of k indicate that clones tend to clump together in infections, whereas high values of k indicate that clones are randomly distributed among infections, in which case the negative binomial approximates the Poisson distribution.

Two studies have employed this method with differing results; one showed strong support of LMC predictions (Read et al. 1995) and one showed no relationship between prevalence and sex ratio (Shutler et al. 1995). Shutler and Read (1998) suggest a number of ecological and physiological factors that could account for the disparity in

the results, including deviations from the assumed negative binomial distribution of clones among infections. These studies lacked any direct estimate of clonal diversity to predict sex ratio independently from prevalence, and also may have failed to detect cryptic taxonomic diversity among the parasites (Martinsen et al. 2008).

We present a study of the relationship between prevalence, genetic diversity and gametocyte sex ratio using *Plasmodium mexicanum*, a malaria parasite of lizards in California USA. The study allowed us to seek the links between these three variables and determine whether prevalence is a viable alternative to clonal diversity for testing sex ratio predictions. The study also allowed us to test the assumption of a negative binomial distribution of clonal numbers and to obtain an empirically determined value of the dispersion parameter k for the best fit to this distribution (the estimates of k used by Read et al. 1995 were roughly based on estimates from helminth parasites). Our study system has the advantages of established molecular techniques for direct estimation of clonal diversity as well as a detailed knowledge of the parasite and host biology such as prevalence rates over time (Vardo and Schall 2007, Schall and St. Denis 2013), estimates of male gametocyte fecundity (Neal 2011), data on natural course of infection (Bromwich and Schall 1986) and evidence of genetic variation for sex ratio (Neal and Schall 2010, 2014). We take advantage of data and samples collected from the same set of local sites at our study area in northern California over a 30-year period to identify sites with chronically high and low parasite prevalence. Using direct measures of sex ratio and clone numbers at these sites, we test two predictions: 1) infections at high prevalence sites will have greater genetic diversity and more clones per infection, and 2) this difference in parasite distribution among hosts will drive infections at high prevalence

sites to have less female-biased sex ratios on average (regardless of whether sex ratios are facultatively determined within each infection or locally adapted *sensu* Schall 2009).

Methods

Sample collection and selection

Plasmodium mexicanum in its lizard and insect host has been studied since 1978 at the University of California Hopland Research and Extension Center (HREC) near the town of Hopland in Mendocino County California (Vardo and Schall, 2007; Schall and St. Denis, 2013). Details on the natural history of this system are presented by Schall (1996). Lizards are captured using a fishing pole with attached noose, and a small sample of blood is taken before the lizard is released at its point of capture. Blood samples are used to make thin blood smears for processing with Giemsa stain. After 1996, a small amount of blood has been taken and stored frozen on filter paper for genetic analysis.

This research program has included nearly annual sampling of various sites at HREC and has revealed that while the prevalence of the parasite varies among years (Schall and St. Denis 2013), some sites consistently have a higher proportion of infected hosts than others (Schall and Marghoob, 1995, Vardo and Schall 2007). For this study, we selected sites with consistently high or low prevalence of *P. mexicanum* in the lizard host. These sites were selected from those that were sampled for at least 4 years during the period between 1980 and 2010 (not all sites are sampled each year, especially those sites that consistently have low prevalence). Using recorded data on the lizards captured and their infection status, we determined the proportion of lizards that were infected for

each site each year. For each year, we ranked sites by the proportion of lizards infected and scored them as being either above or below the median prevalence for that year. We then summed the number of years above vs. below the median for each site. We also calculated the overall percentage of lizards infected at each site over all years in which that site was sampled ($[\text{total infected lizards caught } 1980 - 2010] / [\text{total lizards caught } 1980 - 2010]$). We scored sites as “high prevalence” if they had at least three times as many years above the median prevalence as below and at least 20% of lizards infected overall. Three sites met these criteria (Table 5). Low prevalence sites had at least three times as many years below the median yearly prevalence as above and less than 10% of lizards infected overall. Nine sites met these criteria (Table 5). These criteria were chosen to ensure sustained differences in prevalence among the sites and so that a sufficient sample size was available (especially from low prevalence sites) for a meaningful comparison.

Data (sex ratios and genotyping data; see below) were collected for all available low prevalence site infections and a comparable number of infections from high prevalence sites, matched by year (i.e. if 10 low prevalence infections were available from 2005, the first ~10 high prevalence infections from 2005 - split equally among the 3 sites - were used). This procedure ensures that we did not compare data from low prevalence sites in the high prevalence years with data for high prevalence sites during years with lower overall prevalence for all sites.

Based on the geographical distribution of *P. mexicanum* at HREC, the high prevalence sites were grouped in a central location and low prevalence sites were more

dispersed. All sites are 0.1 – 6 km apart, and sites with different prevalence (high vs. low) are 0.5 – 3.75 km apart. *Plasmodium mexicanum* is genetically differentiated at sites less than 1 km apart (Fricke et al. 2010), suggesting that the distance between these sites may be sufficient for locally adapted traits to develop.

Sex ratio and gametocytemia

Sex ratio and gametocytemia counts were made on Giemsa stained slides viewed on a light microscope at 1000x magnification. To determine sex ratio (proportion male), mature gametocytes were scored as male or female until 100 were counted or for one hour. Both investigators performed counts, and overlapped on a sample of 14 infections to ensure consistency. Gametocytemia was assessed by noting the number of gametocytes (immature, male and female) in a sample of 1000 erythrocytes from different areas of the blood smear. An association between gametocytemia and sex ratio has been observed previously for this species (Schall 2000, Neal and Schall 2014), so it was included as a covariate in the present analysis (see below).

Genetic diversity

All infections in this study with available dried, frozen blood dots were genotyped previously; these include data published by Vardo and Schall (2007; infections from 1996 – 2005), Schall and St. Denis (2013; infections from 1995 and 2006) and Neal and Schall (2014; infections from 2008 – 2010). Genotyping was performed for 4 microsatellite loci- Pmx306, Pmx732, Pmx747 and Pmx839- using fluorescently labeled PCR primers and conditions described by Schall and Vardo (2007). The PCR product was diluted and

processed through an ABI 3730xl DNA analyzer. The parasite is haploid in vertebrate blood (only the ephemeral zygote is diploid in the parasite's life cycle), so each peak seen on a pherogram output from the analyzer corresponds to one clone (at least for that marker) in an infection. Differences in the number of clones per infection identified by these loci have previously been correlated with variation in life history traits (Vardo-Zalik and Schall 2009), so it is likely that the information revealed by them is meaningful for the evolution of life history traits. Genetic data were not available for all infections, particularly those sampled prior to 1996. Seventy-four of the infections selected for sex ratio counts had available genotyping data for at least one locus (49 high, 25 low); these data were used for a general comparison of genetic diversity at high vs. low prevalence sites.

Analysis

All analysis was performed in R version 3.0.2. Sex ratio data did not differ significantly from a normal distribution ($P=0.198$, Shapiro-Wilk test), and no infections with less than 50 gametocytes counted were included in the analysis. ANOVA tested for differences in sex ratios among the three high prevalence sites to determine whether it was possible to combine data from multiple sites. The same was not repeated for low prevalence sites due to the low sample sizes at some sites. ANCOVA was used to test the relationship between sex ratio and prevalence (high vs. low) with gametocyte gametocytemia as a covariate. Gametocytemia was cube root transformed prior to analysis to decrease skew in the data. Observed sex ratios were also compared with those

predicted by the derivation of Read et al. (1995) based on an assumption of a negative binomial distribution among hosts (above).

Five summary statistics were used to compare genetic data from high and low prevalence sites. (1) The minimum number of clones in an infection, calculated as the maximum number of alleles observed at any one locus. GLM was used to test whether the number of clones differed between high and low prevalence sites. (2) The number of alleles. Because more infections were genotyped from high prevalence sites than low, we used rarefaction, a procedure often used to compare species numbers at sites with different numbers of individuals sampled (Gotelli and Colwell 2009). Rarefaction involves repeatedly drawing the number of individuals in the smaller sample from the larger sample and comparing the number of species (alleles) observed in these subsamples with the number observed in the small sample. For this study, we used sample-based rarefaction, meaning that the “individuals” drawn from the larger sample were all of the alleles contained in an infection. For example, locus Pmx306 had 24 low prevalence site infections and 45 high prevalence site infections. We therefore drew 100 samples of 24 infections from the high prevalence infections (without replacement) and compared the distribution of allele numbers observed in these 100 samples with the number of alleles observed in the 24 low prevalence site samples. (3) Expected heterozygosity, calculated as $1 - \sum p_i^2$ where p_i is the frequency of the i th allele in the population. (4) Frequency of rare alleles in multi-clone infections. Clones with low parasite density within an infection could be missed when viewing electropherograms (Vardo and Schall 2007). A common approach to correct for this potential bias has been to include only alleles whose peaks are at least 1/3 the height of the predominant peak in

analysis (Anderson et al. 2000, Vardo and Schall 2007). Instead, we looked at the ratio of small secondary peaks to large secondary peaks at high vs. low prevalence sites. Our rationale is that if small ($< 1/3$ predominant peak) and large peaks are equally common at high and low prevalence sites, it is likely that undetectable peaks are also equally common and therefore should not affect our comparison of clone numbers (i.e. very rare clones are missed at both sites equally). (5) The variance in allele size using a Levene/Brown-Forsythe test. Microsatellite alleles that are more similar in size are generally thought to be more closely related (Goldstein and Schlotterer 1999), so it is possible that there would be a difference in relatedness of the alleles at high vs. low prevalence sites that would be detectable by a difference in their sizes.

Finally, we compared the observed distributions of clones among infections with the negative binomial distribution. Using the microsatellite data and the proportion of uninfected lizards, we obtained empirical estimates of the mean number of clones per host, m , and dispersion, k , for our two different levels of prevalence using the `fitdistr()` function in the R package MASS. We highlight the difference between the mean number of clones per sampled lizard (m), which is used here, and is relevant to fitting this distribution, and the mean number of clones per infected lizard, which is the value being compared in the first analysis described in the previous paragraph and which is the value that is relevant to sex ratio evolution. These two values are closely related if k is relatively constant. We used a chi-squared test to determine the goodness of fit of this distribution after grouping categories with less than 5 observations, following Bliss and Fisher (1953).

Because combining data from multiple sites and years might itself cause deviations from the negative binomial distribution, we also determined m , k , and goodness of fit for data taken from 2 individual sites sampled in 2009 (Water Tank [WT] and Parson's Creek [PC]) and 2 individual sites sampled in 2010 (Middle Lower Horse [MLH] and Gorge [GOR]). These sites were selected because they were the only sites with at least 5 infections with 1 clone and at least 5 infections with >1 clone sampled. For the analysis presented below we refer to these as the "four additional sites." For sites that met these criteria in both 2009 and 2010, only data from the year with more infected lizards were used. We used Fisher's combined probability test to obtain an overall assessment of whether the distribution of clone numbers per host significantly deviated from negative binomial.

Results

Sex ratios

Of 297 infections selected for this study (136 low, 161 high), we were able to locate slides and count at least 50 mature gametocytes for 144 infections: 87 from high prevalence sites and 57 from low prevalence sites (see Table 5 for distribution among sites). Sex ratio counts obtained by the two investigators did not differ ($t = 1.44$, $P = 0.17$, paired t-test), and neither did sex ratios among high prevalence sites ($F = 1.105$, $P = 0.336$, ANOVA) so counts from all high prevalence sites were combined for further analysis, as were counts from low prevalence sites.

Table 5: Summary data for sites at the University of California Hopland Research and Extension Center used in this study. Sites are: Greenhouse (GH), Middle Lower Horse (MLH), and Water Tank (WT). Low prevalence sites are: Coon Lake (CL), Coyote Pens (CP), Fig Tree (FIG), Joe's Dream Pool (JDP), James I (JI), Little Buck (LBUCK), Poor Ranch (POOR) and Watershed (WS); nyears is the number of years lizards were captured at that site; n is number of lizards that were sampled and slides were scanned; ninf is the number of infection of *P. mexicanum* identified at that site overall; %inf is the percent of slides scanned that showed infection; max inf and min inf are the maximum and minimum percent of lizards infected in any one sampling year, with [n] as the number of lizards captured at that site that year; counts indicates the number of infections for which counts of at least 50 gametocytes were made; gen indicates the number of infections for which genotyping information was available.

site	nyears	n	ninf	%inf	max inf [n]	min inf [n]	counts	gen
GH	25	503	109	21.7	62.5 [16]	0 [4]/ 3.7[27]	17	13
MLH	16	1245	260	20.9	50 [48]	8.6 [58]	27	7
WT	21	946	248	26.2	54.7 [84]	0 [11]/7.14[56]	43	29
CL	13	446	21	4.7	16.9 [65]	0 [45]	7	5
CP	18	1117	94	8.4	22.2 [72]	0 [45]	16	5
CR	4	106	8	7.5	16.6 [42]	0 [23]	5	0
FIG	5	190	11	5.8	6.9 [20]	4.8 [84]	5	0
JDP	8	230	4	1.7	3.8 [78]	0 [50]	1	3
JI	12	392	21	5.4	10.7 [65]	0 [52]	12	10
LBUCK	9	120	4	3.3	9.1 [11]	0 [16]	5	0
POOR	7	305	29	9.5	11.9 [42]	8.3 [144]	1	0
WS	12	265	24	9.1	15.4 [13]	0 [13]	5	2

The average (both mean/median) sex ratio of high prevalence sites was 0.44 proportion male; for low prevalence sites it was 0.46. Gametocytemia ranged from 0 to 355 gametocytes per 1000 erythrocytes with a mean of 16.1 and a median of 6. Neither prevalence (high vs. low) nor gametocytemia significantly affected sex ratio, but the interaction between the two did (prevalence: $t = -1.280$; $P = 0.20269$; gametocytemia: $t = -0.274$, $P = 0.78449$; interaction: $t = 2.692$, $P = 0.00798$). It appears the interaction is due to a positive relationship between sex ratio and gametocytemia at low prevalence sites but not high prevalence sites (though this has been observed previously at high prevalence sites as well: Schall 2000; Neal and Schall 2014).

Taking into consideration the range in overall prevalence (Table 5) seen at high ($P = 0.21 - 0.26$) and low sites ($P = 0.017 - 0.095$) and the range in k assumed by Read et

al. (1995), Read et al. (1995)'s derivations predicts sex ratios at low prevalence sites should be 0.006 – 0.07 proportion male and at high prevalence sites should be 0.08 – 0.19 proportion male (without the limitation of low male fecundity, see discussion). Observed sex ratios, though, were much less female biased. In fact, for both high and low prevalence sites, sex ratio was only slightly female biased.

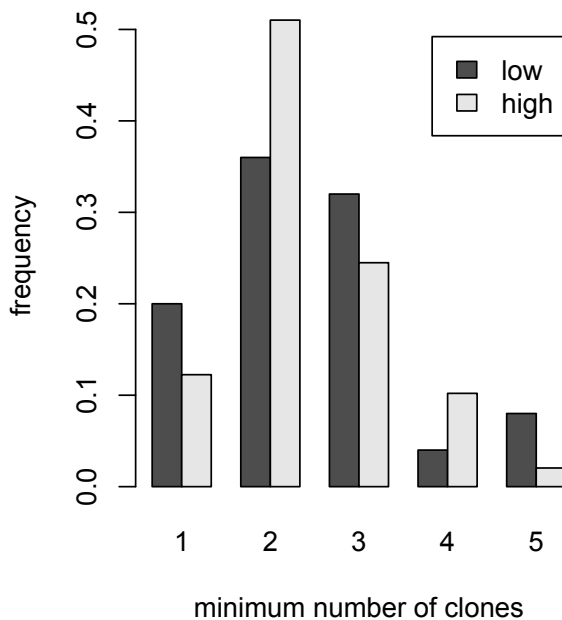


Figure 14: Distribution of clone numbers among infected lizards at low and high prevalence sites.

Genetic diversity

Genotyping data were available for at least 1 locus for 74 infections: 49 from high prevalence sites and 25 from low. The mean number of clones per host (sum[clone numbers]/# lizards sampled) were 0.678 at high prevalence sites and 0.225 at low. For the four additional sites included, the means were 0.313 (GOR, 18/96=18.8% hosts

infected), 0.218 (MLH, 23/179=12.8% hosts infected), 0.593 (WT, 18/54 = 33.3% hosts infected), and 0.247 (PC, 13/93 = 14.0% hosts infected).

Table 6: Comparison of genotypic data at high vs. low prevalence sites. The number of alleles at high prevalence sites is a 95% confidence interval (mean +/- 1.96*standard deviation based on 100 subsamples; n gives the number of infections sampled [low] or subsampled [high]). Heterozygosity is expected heterozygosity. Peak height ratios are the ratio of small:large secondary peaks on electropherograms (see text for more detail).

locus	# alleles			heterozygosity		peak height ratios	
	high	low	n	high	low	high	low
Pmx306	12.3-17.5	14	24	0.902	0.903	0.7272 (16:22)	0.9231 (12:13)
Pmx747	8.7-13.2	11	21	0.841	0.847	0.8571 (6:7)	0.6667 (2:3)
Pmx839	3.3-8.4	6	7	0.811	0.809	0.9375 (15:16)	1.286 (9:7)
Pmx732	8.4-13.4	13	19	0.831	0.845	1.571 (11:7)	0.6667 (2:3)
Av/total	-	-	-	0.846	0.853	0.9231 (48:52)	0.9615 (25:26)

Despite differences in the mean number of clones per host, there was no difference in mean clones per infection (sum[clone numbers]/# infected lizards), which is the value relevant for sex ratio evolution. At high prevalence sites, mean clones per infection is 2.39, and at low sites it is 2.44. When 1 was subtracted from the minimum number of clones per infection, the counts did not differ significantly from a Poisson distribution ($X^2 = 60$, $P = 0.3$), so this transformation was used in comparing clone numbers between prevalence groups. No difference was detected ($P = 0.89$, GLM, family = Poisson; Fig. 14). There also was no difference in the number of alleles observed at high vs. low prevalence sites. For all loci, the number of alleles at the low prevalence sites fell within the rarefied estimates from high prevalence sites (Table 6). Expected heterozygosity is very similar between high and low prevalence sites (Table 6), and there are no significant differences in the variance in allele length ($P > 0.05$ for all 4

loci; Levene/Brown-Forsythe test). There was no consistent difference in the ratio of small to large secondary peaks between high and low prevalence sites: the ratio is higher for high prevalence sites for loci 747 and 732, but lower for loci 306 and 839 (Table 6). Overall, there is no detectable difference in the number of clones per infection or parasite genetic diversity at high vs. low prevalence sites.

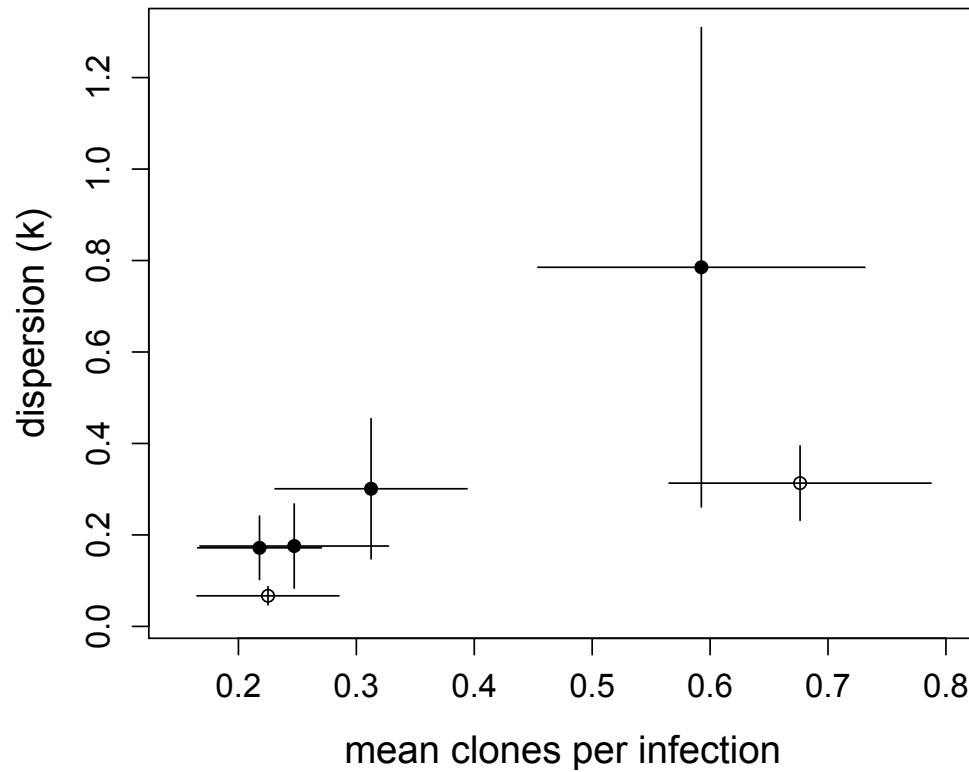


Figure 15: Relationship between the mean (m) and dispersion parameters (k) of the negative binomial distribution fit to counts of clone numbers from infections of the lizard malaria parasite *Plasmodium mexicanum*. Filled points indicate data from 4 individual sites. Open points indicate data from low (left) and high (right) prevalence sites pooled over multiple sites and years. Bars on each point indicate the standard deviation of the parameter estimate.

Fit to the negative binomial

Fitting the negative binomial distribution to the data showed that the dispersion parameter, k , varied among sites, and was higher at sites with a higher mean (Fig. 15).

However, the distributions of clone numbers among infections at both high and low

prevalence sites showed significant deviation from the negative binomial distribution (low: $X^2 = 8.95$, $P = 0.030$; high: $X^2 = 37.4$, $P < 0.0001$), as did those from the additional four individual sites ($X^2 = 24.2$, $df = 8$, $P = 0.00215$, Fisher's combined probability test; P-values for these sites individually are 0.05435 [MLH], 0.227 [GOR], 0.0649 [WT], and 0.00707 [PC]). All sites and grouped sites showed a consistent excess of multi-clone infections (MLH: obs = 14, exp = 8.6; GOR: obs = 10, exp = 6.6; WT: obs = 12, exp = 7.5; PC: obs = 12, exp = 5.1; low sites: obs = 20, exp = 12.8; high sites: obs = 43, exp = 26.5).

Discussion

The life history of malaria parasites matches well Hamilton's (1967) image of local mate competition (LMC) in a patchy breeding world. The world for *Plasmodium* is indeed divided into patches (hosts) and mating between sex cells can take place only among the gametocytes within a single blood meal taken by the insect host. The number of parasite genotypes that will compete for mates in the blood meal is determined by the clonal diversity of the parasite within the vertebrate host that was the source of that blood meal. Thus, clonal diversity should select for the equilibrium gametocyte sex ratio, either through facultative shifts within the vertebrate (phenotypic plasticity in sex ratio) or by a locally adapted sex ratio based on the prevailing clonal diversity at a local site. Either mechanistic path should lead to less biased sex ratios in populations with more clones per host on average. Although molecular markers are now available for the human malaria parasites that would allow direct estimates of clonal diversity and inbreeding, estimation via sex ratio would be less expensive and removes the need for determining relevant

scaling (West 2009). Thus, the model of LMC would provide an example of evolutionary theory being useful for public health efforts.

The Read et al. (1995) study highlighted a third trait, parasite prevalence, that should also relate to clonal diversity and allow testing of LMC predictions for species that lack established genetic markers. This relationship depends on the likely fit of clone numbers among infections to the negative binomial distribution with its parameter k , a measure of the association of clones. Read et al. (1995) thus modeled the relationship between local parasite prevalence and likely clonal diversity, concluding that higher prevalence will be associated with higher clonal diversity, and this would in turn select for less female biased sex ratios. Evidence indicating a close fit of local prevalence with gametocyte sex ratio among sites was found for avian malaria parasites in the genus *Leucocytozon*, but not for another genus in birds, *Haemoproteus* (Read et al. 1995, Shutler et al. 1995). Unfortunately, neither of these study systems had methods for directly estimating genetic diversity, so it is unclear whether uniformity of clone numbers across prevalence could explain Shutler et al.'s (1995) results. This conflict in the among-site tests provoked this study, and we therefore sought to determine whether there was geographic variation in sex ratio based on prevalence, which could arise from local differences in the average number of clones per infection.

Our results conflicted with every expectation of theory: sex ratio did not differ among sites by prevalence, prevalence and clonal diversity were not associated for any of our measures of clonal diversity, and the distribution of clones among infections showed consistent deviations from the negative binomial distribution. The results therefore conflict so strongly with both the sex ratios predicted by prevalence and the intuitively

pleasing idea of higher clonal diversity in areas with higher transmission and prevalence that an explanation for this conflict should provide interesting insights into the biology of malaria parasites.

Sex ratios, prevalence and clone numbers

Observed sex ratios (0.44 – 0.46 proportion male) were substantially higher than the strong female-bias predicted by prevalence alone (0.006 – 0.19 proportion male). The most likely explanations for this deviation is the low fecundity (number of gametes produced) of male gametocytes. *Plasmodium mexicanum* produces only 1.2 - 3 gametes per male gametocyte, which gives a predicted minimum sex ratio of 0.25 - 0.45 proportion males (using expression given in Hamilton 1967; Neal 2011, Neal and Schall 2014). This minimum prediction far exceeds the prevalence-based prediction, and is therefore argues that a low fecundity likely masks any consequence of parasite prevalence.

However, despite this low fecundity, experimentally-manipulated infections of *P. mexicanum* with high clonal diversity (but no higher than some natural infections) show less female-biased sex ratios than those with lower clonal diversity as predicted by LMC (Neal and Schall 2014). This makes the case that sex ratio variation could indeed exist among sites with sustained differences in parasite prevalence, even if not the high female-bias predicted by the model, but none was observed. We are able to rule out many of the possible explanations outlined by Shutler and Read (1998), including lack of genetic variation for sex ratio (Neal and Schall 2010, 2014b), temporal variation in prevalence (such variation exists, but differences among sites remain; Schall and Vardo 2007), and

clearing of infections in older lizards (not observed; Bromwich and Schall 1986). One of the remaining hypotheses, which relates to the ideas we explore here, is the ‘unconventional clumping’ hypothesis.

It seems likely that sex ratios did not differ at high vs. low prevalence sites simply because average clonal diversity among infections did not differ for high and low sites. High prevalence sites in this study did not have more clones per infection, more alleles, higher heterozygosity, greater variation in allele lengths, or a different proportion of small secondary peaks. In fact, this pattern may be more general; the absence of a consistent positive association between prevalence (or transmission intensity) and rates of multiclonality have been noted by a number of authors (e.g. Gatei et al. 2010, Zhong et al. 2007, Mara et al. 2013, Babiker and Walliker 1997, Vardo and Schall 2007).

The unconventional clumping hypothesis suggests that clones are aggregated among hosts in ways that conflict with the negative binomial distribution and thereby obscure the relationship between prevalence and clonal diversity. Deviations from this distribution would disrupt sex ratio predictions based on parasite prevalence. Such deviations were observed in our data, and appeared to be due to a consistent excess of multi-clone infections (or, conversely, a deficit of single-clone infections). One plausible explanation for this pattern is that not all lizards at a site are either exposed or susceptible to the parasite. If the degree of exposure or susceptibility were continuous, it could cause patterns of aggregation that could likely be accounted for by the negative binomial (Crofton 1971, Anderson and May 1991). However if exposure or susceptibility were discrete, it could cause deviations from this distribution. We feel it unlikely that variation in susceptibility among lizards could account for such a pattern in *P. mexicanum* because

attempts to experimentally infect lizards have a ~100% success rate (Eisen and Schall 2000, Osgood and Schall 2004, Vardo et al. 2007). Heterogeneity in exposure, though, is quite possible. Previous studies have revealed that variation in infection prevalence exists on a very local scale for fence lizards at the HREC (over meters- Eisen and Wright 2001), and this highly localized variability has also been suggested in other species (e.g. *Plasmodium falciparum*, Gaudart et al. 2006). This could also account for similar distributions of clones among infections at high and low prevalence sites: both may have similar very localized spots with high transmission. Low prevalence sites may simply have fewer transmission hotspots.

The deviations from the negative binomial observed in our data could also result from an underestimation of the number of clones per infection. It may seem counterintuitive that having multi-clone infections with more clones would ‘correct’ the problem of too many multi-clone infections, but the effect on the fit distribution would be to increase both the estimated mean and degree of clumping, thereby increasing the number of multi-clone infections expected. Our method of estimating clones per infection (taking the maximum number of alleles at any one locus) is likely to underestimate the true number of clones in some cases because clones may share alleles. The total number of possible clones (assuming all are present have a unique combination of microsatellite alleles) would instead be the product of the number of alleles at each locus (Walliker et al. 1998, Vardo and Schall 2007).

As a final note on the distribution of clones, it is not even necessary that aggregation be inconsistent with the negative binomial to decouple prevalence and clonal diversity: variable aggregation is sufficient. For example, consider two populations, one

with $m = 0.4$ and $k = 0.3$ and the other with $m = 0.8$ and $k = 1.1$. The two populations would differ greatly in the proportion of hosts infected (0.22 vs. 0.45), but the distribution of clones among infected lizards would be strikingly similar (no more than 0.03 proportion different for any number of clones). A positive association between m and k is suggested by our data (Fig. 15) and would result if the forces favoring aggregation were weaker (or those favoring segregation were stronger) in populations with more infections. As an example, premunition, the partial or complete blocking of new clones entering an already infected host (Vardo et al. 2007, de Roode et al. 2005), should promote segregation and may be more influential when a higher proportion of hosts are already infected.

To sum up, sex ratios were not higher at high prevalence sites, contrary to predictions. A similar lack of pattern was observed by Shutler et al. (1995), but not by Read et al. (1995), who observed the predicted relationship. Having considered many of the hypotheses put forth by Shutler and Read (1998) to explain the discrepancy in their results, we find a lack of concordance between prevalence and clone numbers to be the most likely for our data. Fecundity is indeed low for *P. mexicanum*, which almost certainly accounts for deviations from the numeric predictions based on prevalence. However, we also found significant deviations from the negative binomial distribution and evidence suggesting a positive relationship between average clones per infection (m) and the degree of clonal aggregation (inverse of k), both of which could account for the uniformity of clone numbers despite differences in prevalence.

Conclusions

Malaria parasite prevalence is a logical correlate of clonal diversity in theory, but it may not be a reliable one in practice. This is unfortunate for the study of sex ratios in natural populations, because prevalence is a convenient substitute for clonal diversity, especially for less-studied species that lack developed genetic techniques. However, it is also encouraging in that these new results may help resolve the question of why a relationship between sex ratio and prevalence was not seen for *Haemoproteus* (Shutler et al. 1995). In doing so, though, it raises new questions like why the predicted pattern was apparent for *Leucocytozoon*. Is there something about the biology of this genus (Read et al. 1995's study was not limited to one species) that allows for the intuitive link between prevalence and clonal diversity? If so, what makes it break down in other genera, like *Plasmodium*?

These questions, and a general understanding of how prevalence and transmission intensity influence clonal diversity, are themselves of great significance for public health. This importance is indicated by the number of researchers who have now explored the relationships between prevalence/transmission rates and infection complexity using modern molecular techniques (e.g. Anderson et al. 2000, Iwagami et al. 2009, Gatei et al. 2010, Mara et al. 2013). Our study adds to the growing body of literature suggesting that neither transmission rate nor parasite prevalence is a consistent predictor of clones per infection. We additionally suggest that the strength of factors influencing clonal aggregation among hosts may vary with prevalence, and, if better understood, could allow for better prediction of the incidence of multiple infections.

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References

- Anderson RM, May RM (1991) Infectious diseases of humans. Oxford University Press, Oxford.
- Anderson TJ, Paul RE, Donnelly CA and Day KP (2000) Do malaria parasites mate non-randomly in the mosquito midgut? *Genetical Res.* 75: 285-296.
- Babiker HA, Walliker D (1997) Current views on the population structure of *Plasmodium falciparum*: implications for control. *Parasitol. Today* 13: 262-267.
- Bull JJ, Charnov EL (1988) How fundamental are Fisherian sex ratios? *Oxford Surv. Evol. Biol.* 5: 96-135.
- Bliss CI, Fisher RA (1953) Fitting the negative binomial distribution to biological data. *Biometrics* 9: 176-200.
- Clark AB (1978) Sex ratio and local resource competition in a prosimian primate. *Science* 201: 163-165.
- de Roode JC, Helinski MEH, Anwar MA, Read AF (2005) Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am. Nat.* 166: 531-542.
- Dye C and Godfray HCF (1993) On sex ratio and inbreeding in malaria parasite populations. *J. of Theor. Biol.* 161: 131-134.
- Edwards AWF (1998) Natural selection and the sex ratio: Fisher's sources. *Am. Nat.* 151: 564-569.

Edwards AWF (2000) Carl Düsing (1884) on the regulation of the sex-ratio. *Theor. Pop. Biol.* 58: 255-257.

Eisen RJ, Schall JJ (2000) Life history of a malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *P. Roy. Soc. Lond. B* 267: 793-799.

Eisen RJ and Wright NM (2001) Landscape features associated with infection by a malaria parasite (*Plasmodium mexicanum*) and the importance of multiple scale studies. *Parasitology* 122: 507-513.

Fisher RA (1930) The genetical theory of natural selection. Dover Publications, New York.

Fricke JM, Vardo-Zalik AM, Schall JJ (2010) Geographic genetic differentiation of a malaria parasite, *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*. *J. Parasitol.* 96: 308-313.

Garnder A, Reece SE, West SA (2003) Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *J. Theor. Biol.* 223: 515-521.

Gatei W, Kariuki S, Hawley W, ter Kuile F, Terlouw D, Phillips-Howard P, Nahlen B, Gimnig J, Lindblade K, Walker E, Hamel M, Crawford S, Williamson J, Slutsker L, Shi YP (2010) Effects of transmission reduction by insecticide-treated bed nets (ITNs) on parasite genetics population structure: I. The genetic diversity of *Plasmodium falciparum* parasites by microsatellite markers in western Kenya. *Malaria J.* 9: 353.

Gaudart J, Poudiougou B, Dicko A, Ranque S, Toure O, Sagara I, Diallo M, Diawara S, Ouattara A, Diakite M, Doumbo OK (2006) Space-time clustering of childhood malaria at the household level: a dynamic cohort in a Mali village. *BMC Public Health* 6: 286.

Goldstein DB, Schlotterer C (eds.) (1999) Microsatellites: evolution and applications. Oxford University Press, Oxford.

Gotelli NJ and Colwell RK (2009) Estimating species richness. In: Magurran AE, MacGill BJ (eds.) *Biological diversity: frontiers in measurement and assessment*. Oxford University Press, Oxford pp 39-54.

Hamilton WD (1967) Extraordinary sex ratios. *Science* 156: 477-488.

Hartl DL (1971) Some aspects of natural selection in arrhenotokous populations. *Am. Zool.* 11: 309-325.

Mara SE, Silué KD, Raso G, N'guetta SP, N'goran EK, Tanner M, Utzinger J, Ding XC (2013) Genetic diversity of *Plasmodium falciparum* among school-aged children from the Man region, western Côte d'Ivoire. *Malaria J.* 12: 419.

- Neal AT (2011) Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 138: 1203-1210.
- Neal AT, Schall JJ (2010) Gametocyte sex ratio in single-clone infections of the malaria parasite *Plasmodium mexicanum*. *Parasitology* 137: 1851-1859.
- Neal AT, Schall JJ (2014) Testing sex ratio theory with the malaria parasite *Plasmodium mexicanum* in natural and experimental infections. *Evolution* (in press).
- Neal AT and Taylor PD. Local mate competition and transmission bottlenecks: a new model for understanding malaria parasite and other sex ratios. *J. Theor. Biol.* (in review)
- Osgood SM, Schall JJ (2004) Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* 128: 23-29.
- Paul RE, Day KP (1998) Mating patterns of *Plasmodium falciparum*. *Parasitol. Today* 14: 197-202.
- Pickering J, Read AF, Guerrero S, West SA (2000) Sex ratio and virulence in two species of lizard malaria parasites. *Evol. Ecol. Res.* 2: 171-184.
- Read AF, Anwar M, Shutler D, Nee S (1995) Sex allocation and population structure in malaria and related parasitic protozoa. *P. Roy. Soc. Lond. B* 260: 359-363.
- Read, AF, Day KP (1992) The genetic structure of malaria parasite populations. *Parasitol. Today* 8: 239-242.
- Read AF, Narara A, Nee S, Keymer AK, Day KP (1992) Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Read AF, Taylor LH (2001) The ecology of genetically diverse infections. *Science* 292: 1099-1102.
- Schall JJ (2000) Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.
- Schall JJ (2009) Do malaria parasites follow the algebra of sex ratio theory? *Trends Parasitol.* 25: 120-123.
- Schall JJ, Marghoob AB (1995) Prevalence of a malaria parasite over time and space: *Plasmodium mexicanum* in its vertebrate host, the western fence lizard *Sceloporus occidentalis*. *J. Anim. Ecol.* 64: 177-185.

- Schall JJ, St. Denis KM (2013) Microsatellite loci over a thirty-three year period for a malaria parasite (*Plasmodium mexicanum*): bottleneck in effective population size and effect on allele frequencies. *Parasitology* 140: 21-28.
- Schall JJ, Vardo AM (2007) Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Mol. Ecol. Notes* 7: 227-229.
- Shutler D, Bennett GF, Mullie A (1995) Sex proportions of *Haemoproteus* blood parasites and local mate competition. *P. Natl. Acad. Sci. USA* 92: 6748-6752.
- Shutler D, Read AF (1998) Local mate competition, and extraordinary and ordinary blood parasite sex ratios. *Oikos* 82: 417-424.
- Sowunmi A Gbotosho GO, Happi CT, Folarin OA, and Balogun ST (2009) Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitol. Int.* 58: 438-443.
- Taylor PD (1981) Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature* 291: 64-66.
- Vardo-Zalik AM (2009) Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host. *Int. J. Parasitol.* 39: 1573-1579.
- Vardo AM, Kaufhold KD, Schall JJ (2007) Experimental test for premunition in a lizard malaria parasite (*Plasmodium mexicanum*). *J. Parasitol.* 93: 280-282.
- Vardo AM, Schall JJ (2007) Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Mol. Ecol.* 16: 2712-2720.
- Vardo-Zalik AM, Schall JJ (2009) Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* 90: 529-536.
- West SA (2009) Sex allocation. Princeton University Press, Princeton.
- West SA, Reece SE, Read AF (2001) Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends Parasitol.* 17: 525-531.
- West SA, Smith TG, Nee S, Read AF (2002) Fertility insurance and the sex ratios of malaria and related Hemosporid blood parasites. *J. Parasitol.* 88: 258-263.

Zhong D, Afrane Y, Githeko A, Yang Z, Cui L, Menge DM, Temu EA, Yan G (2007)
Plasmodium falciparum genetic diversity in western Kenya highlands. Am. J. Trop. Med.
Hyg. 77: 1043-1050.

CHAPTER 6: Life history focus on a malaria parasite: linked traits and variation among genetic clones

Allison T. Neal and Joseph J. Schall

Abstract

Life history theory has long been a major campaign in evolutionary ecology, but has typically focused only on animals and plants. Life history research on single-celled eukaryotic protists such as malaria parasites (*Plasmodium*) will offer new insights into the theory's general utility as well as the parasite's basic biology. For example, parasitologists have described the *Plasmodium* life cycle and cell types in exquisite detail, with little discussion of evolutionary issues such as developmental links between traits. We measured 10 life history traits of replicate single-genotype experimental *Plasmodium mexicanum* infections in its natural lizard host to identify groups of linked traits. These 10 traits formed 4 trait groups: "Rate/Peak" merges measures of growth rate and maximum parasitemia of infections; "Timing" combines time to patency and maximum parasitemia; "Growth Shape" describes the fit to an exponential growth curve; and "Sex Ratio" includes only the gametocyte sex ratio. Parasite genotype (clone) showed no effect on the life history trait groups, with the exception of gametocyte sex ratio. Therefore, variation in most life history traits among infections appears to be driven by environmental (individual host) effects. The findings support the model that life history traits are often linked by developmental constraints. Understanding why life history traits of *Plasmodium* are linked in this way would offer a new window into the evolution of the parasites, and also should inform public health efforts to control infection prevalence.

Introduction

The partitioning of assimilated resources into growth, maintenance, and reproduction, and the timing of those developmental decisions, define an organism's life history. Important life history traits include age at first reproduction, the number and size of offspring, the frequency of reproductive episodes, and for sexually reproductive species, the allocation of effort to male and female offspring. Theoretical and empirical study of life histories has led to insights on how natural selection shapes complex trade-offs resulting from conflicts in resource and time allocation as well as sometimes obscure developmental constraints (Reznick and Endler 1982; Eisen and Schall 2000; Stearns 1992; Charnov 1993; Gotelli 2008). This research approach has been profoundly successful for a great variety of large multi-cellular species, but many life history traits studied in such organisms also have close analogues for single-celled protists such as malaria parasites (*Plasmodium* and related genera, Martinsen et al 2008). For more than a century, malaria researchers have focused on the parasite's life cycle, describing each cell type in this cycle in detail, but with remarkably little emphasis on evolutionary questions. A life history approach, however, leads to unexpected insights in the study of malaria parasites. For example, the parasite's life history traits intersect with competition among clonal lines within mixed-genotype infections (Taylor et al 1997; Vardo-Zalik and Schall 2009; Pollitt et al 2011), resource availability and environmental (host) variation (Graves et al 1984; Pollitt et al 2011), potential vaccines (Buckling et al 1997, 1999; Barclay et al 2012), and virulence (Mackinnon and Read 1999a, 1999b).

Malaria parasites have a two-host life cycle, alternating between a vertebrate host and an insect vector. Within both hosts, an infection may harbor a single genetic clone of asexually reproducing cells, or two to several such clones (Read and Day 1992; Paul and Day 1998; Vardo and Schall 2007). Asexually replicating cells (schizonts) undergo cycles of replication in the vertebrate host in which each schizont produces several to many new cells, which then develop into a feeding stage (trophozoite). Trophozoites either continue asexual replication as schizonts or develop into non-reproducing dimorphic male and female sex cells, or gametocytes. Production of gametocytes therefore necessarily reduces asexual growth (Fig. 16). Only the gametocytes survive transmission to the insect host where they initiate the sexual cycle, followed by asexual production of the transmission stages (sporozoites). All parasites stages are haploid with the exception of the ephemeral zygotes that undergo meiosis to yield haploid sporozoites. The parasites therefore reproduce as clones of cells that could well coordinate their efforts to maximize reproductive success of the group (Eisen and Schall 2000; Reece et al 2009). Indeed, clones within both hosts have characteristics that would be expected of individual organisms such as apoptosis (Al-Olayan et al 2002) and, potentially, self-sacrifice to trigger down-regulation of the immune response (Guilbride et al 2012), so each clone appears to function as a multicellular organism.

Many events of *Plasmodium* biology in the vertebrate host mirror life history traits of multi-cellular organisms: asexual proliferation corresponds to somatic growth and maintenance of future reproductive ability, maximum asexual parasitemia is the maximum body size, timing of first production of gametocytes is the age of maturity, and number of gametocytes produced is the investment in male and female offspring.

Although measuring the precise allocation of resources to growth vs. reproduction in multi-cellular organisms is difficult, counts of asexual stage parasites and gametocytes make evaluating investment in these two traits feasible for malaria parasites (Reece et al 2009).

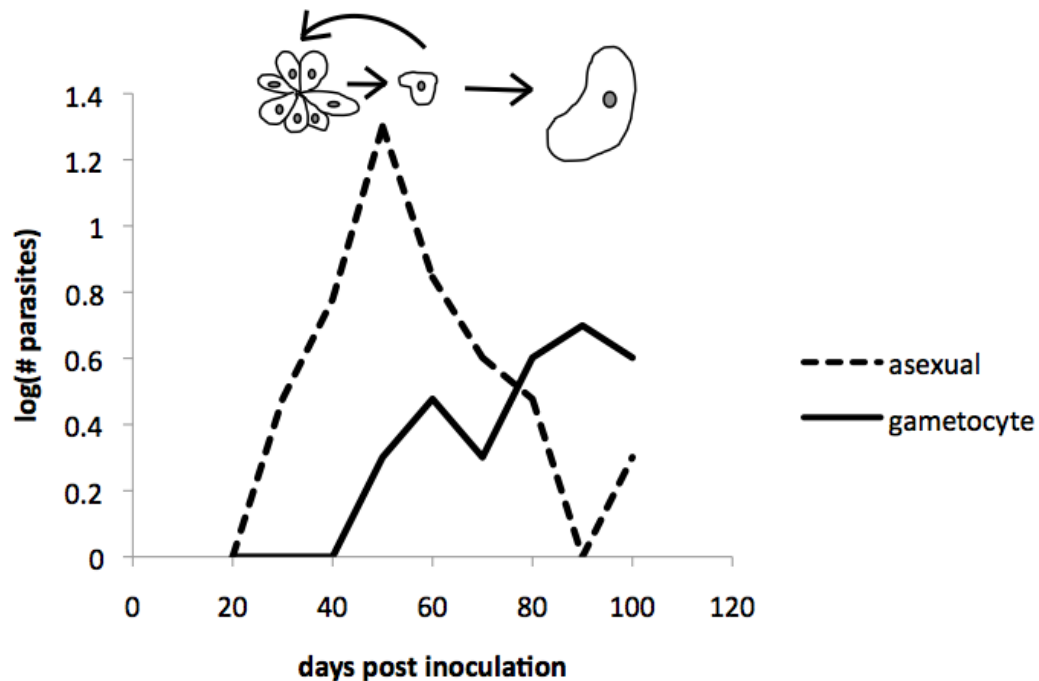


Figure 16: Blood-stage life cycle and life history of the malaria parasite *Plasmodium mexicanum*. At the top of the figure is the life cycle: the parasite reproduces asexually within the blood (arrows at 1), alternating between the single-nucleated trophozoite stage (middle drawing), which develops into the multi-nucleated schizont stage (left, number of nuclei in illustration not meaningful). Schizonts divide to produce multiple new trophozoites. Some trophozoites develop instead into sexual cells (gametocytes, right drawing, arrows at 2). The lower portion of the figure shows parasite counts from one infection in this study. The life cycle is aligned with the plotted data to show which type of cell (1- asexual vs. 2- sexual) is more prevalent in the blood at that time. Shown in the plot are the log transformed number of parasites including asexually replicating stages (schizonts and trophozoites) and gametocytes over 100 days, the normal period for reproduction in the seasonal environment of this parasite-host system. 1 was added to all counts before transformation to avoid undefined values. Counts are from an infection in this study that was experimentally initiated with blood from donor 3.

Understanding how life history traits are linked for malaria parasites is important for public health efforts. For example, the rate of increase in the asexually reproducing

cells in the vertebrate's blood is associated with virulence (Mackinnon and Read 1999a), so identifying links between asexual replication rate and other life history traits would help predict how different treatment or prevention strategies select for increased or decreased virulence (e.g. Barclay et al 2012). Also, gametocyte sex ratio influences transmission success, and sex ratio theory posits that male gametocyte fecundity (production of gametes) drives equilibrium sex ratio in single-genotype infections (Neal 2011; Hamilton 1967). The rate of gametocyte production could alter the number of male gametes that can be produced, thus linking to gametocyte sex ratio and transmission success.

To pursue these issues, we examined the relationships among life history traits for *Plasmodium mexicanum* Thompson and Huff 1944, a malaria parasite of lizards in northern California. We induced experimental infections containing only a single clone of parasite cells and followed the infections for 100 days, roughly the period available for the full course of an infection of this parasite in its seasonal environment (Bromwich and Schall, 1986). A previous study on *P. mexicanum* found life history traits were linked and varied among experimental infections initiated with blood from different donor lizards (Eisen and Schall 2000). However, the number of clones in the experimental infections was not assessed, so any patterns could have been a result of clonal interactions rather than genetic variation for the traits. The life histories of malaria parasites are influenced by clonal interactions in the vertebrate blood (Taylor et al 1997; de Roode et al 2005; Reece et al 2009; Vardo-Zalik and Schall 2009), so our experiment eliminated the confounding factor of clonal interactions such as competition. Therefore, using replicate single-genotype infections we sought to determine 1) which life history

traits were closely associated for *P. mexicanum* and 2) whether groups of associated traits showed evidence of genetic variation.

Materials and Methods

Infected and not infected western fence lizards (*Sceloporus occidentalis* Baird and Girard 1852) used for this study were collected at the University of California Hopland Research and Extension Center (HREC, Hopland, Mendocino County, CA; Schall 1996; Schall and St. Denis 2013) in late May to early June 2010, and each lizard was marked with a unique toe clip combination. A few drops of blood from a toe clip were stored frozen on filter paper for genetic analysis and a drop was used to make a thin smear for processing with Giemsa stain. Infected lizards were captured at sites where *P. mexicanum* has long been most common at the HREC (5 – 18% of lizards infected in 2010), and thin smears were scanned for 3 minutes to identify infected individuals. DNA was extracted from the stored blood dots of infected lizards using the Qiagen DNeasy Kit (Qiagen Sciences, Germantown, Maryland, USA) and subjected to PCR for 4 microsatellite loci using primers and conditions developed by Schall and Vardo (2007). We measured the density of erythrocytes using a hemocytometer and counted the number of asexual stage parasites (trophozoites and schizonts) per 1000 erythrocytes to estimate the density of asexual stage parasites per μL of blood. Eight infections were selected that had high asexual parasite density (8 – 105 asexuals/1000 erythrocytes; 535,000 – 1,580,000 cells/ μL ; 9,900 – 81,885 asexuals/ μL blood) and a single allele at all 4 microsatellite loci (scored therefore as single-clone infections; Anderson et al (2010) showed that 4 microsatellites were sufficient to differentiate approximately 90% of *P.*

falciparum clones). These infected lizards served as blood donors to initiate replicate infections in previously not infected lizards.

Not infected lizards were collected from sites where *P. mexicanum* has been very rare or absent over many years at HREC (0 – 2% of lizards infected in 2010). All lizards in which new infections were initiated were males at least 60 mm in length (snout to vent, excluding tail), and DNA was extracted from their blood and subjected to PCR to amplify a short segment of the cytochrome *b* gene to confirm they were not already infected.

This protocol can identify infections that are not patent upon microscopic examination (Perkins et al 1998). Infected blood from each donor was diluted with PBS, and each of 8 recipient lizards was injected intra-peritoneally with 20 μ L of the mix containing 1×10^5 asexual parasites. Recipient lizards were housed outdoors in vector-proof cages for the first 60 – 70 days of the experiment and were moved indoors for the remaining 30 – 40 days for logistical reasons. Lizards were fed each day with crickets and mealworms to satiation and blood samples were used to make thin smears every 10 days for 100 days.

This sampling regime was based on previous experience. Naturally infected lizards have been studied at the field site for more than 3 decades (Schall and St. Denis 2013), including mark-recapture studies over the entire lifespan of infected lizards (Bromwich and Schall 1986). Sand fly vectors become active by June, new infections go through their annual cycle during the next ~ 100 days, and the blood parasite density (parasitemia) drops during the host's winter brumation and then rebounds the next spring (Bromwich and Schall 1986; Schall and Marghoob 1995). Events in *P. mexicanum* infections, such as rate of increase in parasitemia and production of gametocytes, take far longer than for the malaria parasites of mammals. Validation studies early in the project

sampled infections from every hour to 10 days and determined that a 7-10 day sampling program provided clear data on the course of infection. This sample program has been successful for prior studies, including determining infection growth rate, first patency, and gametocyte sex ratio (Eisen and Schall 2000; Eisen 2000; Osgood et al 2003; Osgood and Schall 2004; Vardo-Zalik and Schall 2008, 2009; Neal and Schall 2010; Ford and Schall 2011). Therefore, we followed the experimental infections for 100 days, sampling the infection every 10 days.

Ten life history traits measured were: Day post inoculation for patency of (1) asexual (schizonts and trophozoites) and (2) gametocytes in the blood while counting 1000 erythrocytes. Peak (3) asexual and (4) gametocyte parasitemia per 1000 erythrocytes for any sample period. The number of days since inoculation for maximum (5) asexual and (6) gametocyte parasitemia. Growth rate of (7) asexual and (8) gametocyte parasitemia in the blood. Growth rates were measured as (a) maximum parasitemia/number of days from inoculation to day of that maximum; (b) maximum parasitemia/number of days since the infection became patent to maximum; (c) maximum growth in parasitemia over a single 10 day period between samples. These measures were tightly correlated ($r^2 > 0.84$), so only the third (c) is included the analysis. (9) The degree to which growth was exponential. The most simple growth would be exponential, with a constant rate of replication of schizonts, and deviations from this pattern would indicate more complex forces at work, such as interaction with the immune system, production of gametocytes, and even apoptosis to regulate somatic growth. To quantify this, a linear model was fit to \ln transformed parasitemia for the days from patency to maximum parasitemia (provided at least 3 sample points were available). The r^2 value of

this relationship was then used as a measure of the fit to an exponential growth model.

(10) Gametocyte sex ratio. Mature gametocytes (based on size and shape; Schall 1989) were scored as male or female until 100 were counted or for 1 hr to calculate sex ratio as proportion male gametocytes. Studies on natural infections (Bromwich and Schall 1986; Schall 1989) found that gametocyte sex ratio was constant in most infections (but varied among infections). For experimental infections, gametocyte sex ratio appears to stabilize by day 80 (Osgood et al 2002, 2003; Osgood and Schall 2004; Neal and Schall 2010), therefore gametocyte sex ratios were determined at day 80 post inoculation.

Principal components analysis was used to cluster these 10 traits. For 9 infections, the lizard host died early or parasites did not become patent in the blood until late in the infection, resulting in insufficient data for estimation of certain life history traits (e.g. gametocyte patency, exponential growth fit). Sex ratios were only included in the analysis if at least 50 mature gametocytes were counted. Because this further limited the sample size (55/64 infections had complete data without sex ratio, 37/64 did with sex ratio), the analysis was run with and without sex ratio included. The number of principal components analyzed was determined based on examination of a scree plot (Jolliffe 2002; Gotelli and Ellison 2004). Variables with eigenvector coefficients (loadings) at least 70% of the maximum coefficient for that principal component are reported as contributing to the component, whereas those at least 50% of the maximum are reported as marginally contributing (Jolliffe 2002). Opposite signs of the loadings can be used to highlight tradeoffs between the variables to which they correspond (Gotelli and Ellison 2004). Most of the traits measured were not normally distributed, and in some cases, no transformation was found that could correct the variable to a normal distribution. As a

result, the principal components were also not normally distributed, so we used a permutation test written in R (R Foundation for Statistical Computing, Vienna, Austria) to determine if the first principal components (number based on scree plot, with and without sex ratio) varied significantly among donor groups or genotypes (see results for explanation of genotypes). The permutation test extracted F from ANOVA for the observed data, then randomly shuffled the principal components 1000 times (10,000 times if the resulting P was < 0.1), storing F for each permutation to create a randomized F distribution. The observed F was then compared with the randomly generated F distribution to obtain P. The principal components obtained using the data from before the lizards were moved inside or all together gave similar results. Only the results from the full study are reported here.

Results

Of the 8 donor infections used in this study, there were 7 unique genotypes. Two infections had identical alleles at all 4 microsatellite loci, and the two lizards with matching alleles at all loci were collected from 2 adjacent (and in contact) sites. Based on the frequencies of these 4 alleles at the 2 sites during 2010 (0.138, 0.121, 0.517, 0.097), the probability of having this genotype and not sharing a recent ancestor is very low (product = 0.00084). It is therefore likely that these 2 infections derive from the same clonal lineage. Subsequent analysis was performed separately with both donor (N = 8) and genotype (N = 7) as independent variables.

Table 7 shows pairwise correlations among the 10 traits measured. Many of these traits are tightly correlated, so the Principal Component analysis was appropriate to

cluster non-independent traits. Based on the scree plots (Fig. 17), we analyzed 4 principal components for the data including gametocyte sex ratio, which explain 81% of the overall variation, and 3 principle components for the data that excluded gametocyte sex ratio, which explain 87% of the variation.

Table 7: Correlations among traits. Values in bold indicate that the 95% confidence interval does not include 0.

Traits*	aPeak	gPeak	aRate	gRate	aMaxDay	gMaxDay	aPatency	gPatency	SexRat	Fit
aPeak	1	0.77	0.95	0.66	0.17	0.11	-0.24	-0.40	-0.04	0.34
gPeak		1	0.74	0.96	0.11	0.15	-0.30	-0.44	0.062	0.30
aRate			1	0.62	0.13	0.07	-0.20	-0.32	-0.14	0.32
gRate				1	0.11	0.14	-0.32	-0.41	0.11	0.31
aMaxDay					1	0.73	0.37	0.28	0.06	-
gMaxDay						1	0.23	0.14	0.10	0.10
aPatency							1	0.58	-0.12	-
gPatency								1	0.14	0.15
SexRatio									1	0.22
ExpFit										1

* Traits are asexual and gametocyte peak parasitemia (aPeak, gPeak), asexual and gametocyte maximum rate of increase (aRate, gRate), the day on which asexuals and gametocytes reached their maximum/peak (aMaxDay, gMaxDay), the number of days post inoculation that asexual and gametocytes became patent (aPatency, gPatency), the gametocyte sex ratio (SexRat) and the r^2 value from a line fit to \ln transformed parasitemias (Fit).

The first principal component encompasses predominantly rate of increase and peak parasitemia of infections, and will be referred to as “Rate/Peak” (Table 8). The loadings for traits associated with rate and peak were all positive (Table 8). The second principal component, referred to as “Timing”, describes the time to reach peak parasitemia and, to a lesser extent, to become patent, and relevant loadings are again all positive (Table 8). The traits contributing to the first and second principal components are not greatly affected by inclusion of sex ratio in the analysis (Table 8). The third principal component mainly describes sex ratio if sex ratio is included in the analysis (“Sex Ratio”, Table 8). The third principal component if sex ratio is excluded and the

fourth principal component if sex ratio is included both largely describe how well an exponential curve fits the data on the growth phase of the infection (“Growth Shape”, Table 8).

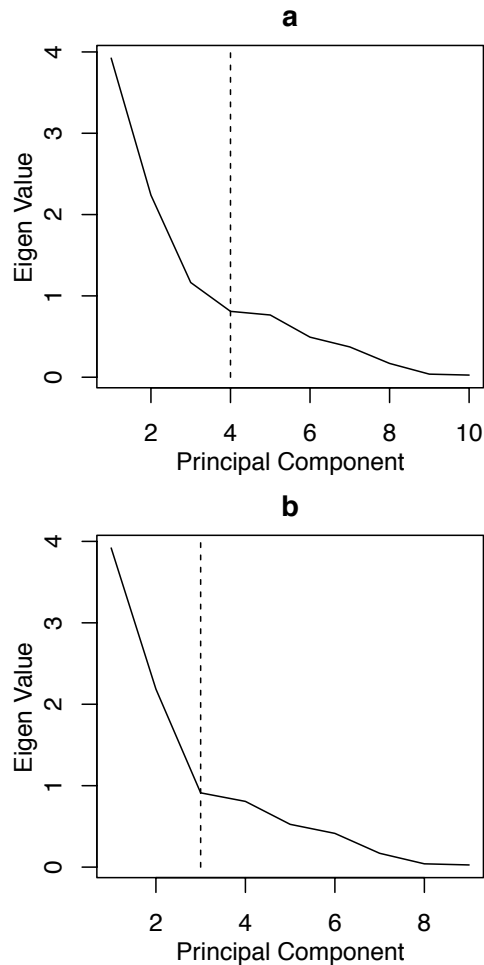


Figure 17: Scree plots with (a) and without (b) sex ratio included. The y-axis shows the percent of the total variation explained (eigenvalue) by each principal component (x-axis). Scree plots are used in principal components analysis for judging the number of meaningful principal components. The plot can be imagined as a mountainside with rubble (scree) at the bottom: the points making up the “mountainside” represent principal components that explain a large proportion of variation, whereas the “scree” are the points that contribute very little to explaining variation. The vertical lines on this plot represent the number of components we selected for analysis based on these plots.

Neither Rate/Peak nor Growth Shape varied among donors or genotypes, whether or not sex ratio was included in the analysis (Fig.18a and Fig.18d, $F < 1.1$, $P > 0.4$, permutation tests). Timing varied among donors if sex ratio was excluded from the

analysis (Fig. 18b, $F = 2.98$, $P = 0.0096$, $N = 55$, permutation), but not if sex ratio was included ($F = 0.649$, $P = 0.722$, $N = 37$, permutation), and did not vary among genotypes ($F < 1.3$, $P > 0.25$, permutation). Sex Ratio showed significant variation among both donors (Fig. 18c, $F = 2.56$, $P = 0.0329$, permutation) and genotypes ($F = 3.10$, $P = 0.0203$, permutation). Sex ratio itself (not the Sex Ratio principal component) also showed significant variation among donors ($X^2 = 15.96$, $P = 0.0255$, GLM with binomial errors) and genotypes ($X^2 = 15.90$, $P = 0.0143$, GLM).

Table 8: Summary of traits measured for experimental infections. Traits correspond to those in Table 7. Average values are expressed as mean (median), and maximum and minimum values and contribution to principal components are also given. Starred principal components indicate results from analysis without sex ratio data. “+” and “-” signs without parenthesis indicate the coefficient in the eigenvector was at least 70% the maximum coefficient for that principal component. Parentheses indicate the coefficient was 50 – 70% of the maximum.

Trait	Av.	Min	Max	N	PC1	PC1*	PC2	PC2*	PC3	PC3*	PC4
aPeak	137 (56)	2	1144	61	+	+					
gPeak	18.2 (11)	1	89	58	+	+					
aRate	7.63 (3.2)	0.2	80.9	61	+	+					
gRate	1.09 (0.65)	0.1	5.6	58	+	+					
aMaxday	77.9 (80)	30	100	61				+	+		
gMaxDay	80 (80)	40	100	58				+	+		
aPatency	33.8 (30)	10	60	61				(+)	+		(+)
gPatency	51.9 (50)	20	80	58	(-)	(-)	(+)	(+)			
SexRat	0.47 (0.48)	0.36	0.58	37		na		na	+	na	
Fit	0.86 (0.903)	0.314	0.989	55					(+)	-	+

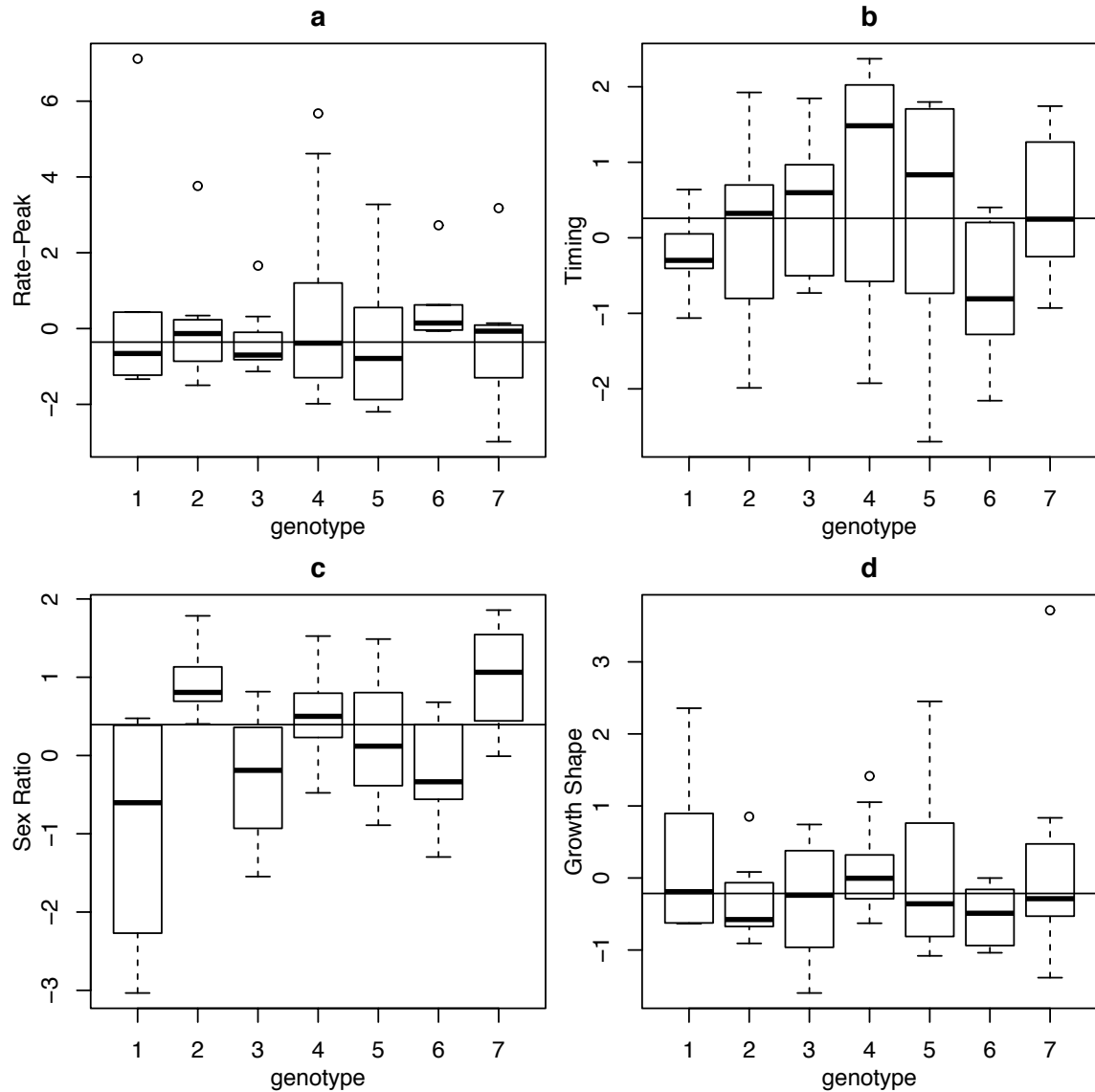


Figure 18: Influence of donor infection (each with a single parasite genetic clone) on combined life history traits of *Plasmodium mexicanum* in its natural lizard host. Shown are distribution of (a) Rate/Peak, (b) Timing, (c) Sex Ratio and (d) Growth Shape principal components for each donor. Data presented are from analysis excluding sex ratio with the exception of plot (c). Donors 4 and 5 have the same microsatellite genotype at 4 scored loci and are therefore assumed to be the same clone. The horizontal line is the overall median. Shown are standard information for a box plot: the middle (bold) bar is the median, the box shows the interquartile range (IQR, range that includes middle 50% of data), the whiskers extend to the highest and lowest data point within $1.5 \times \text{IQR}$ of the box, and points are outliers.

Discussion

We take a life history approach for a malaria parasite, and break down events during the course of an infection in the vertebrate host's blood into traits analogous to those for the life history of multi-cellular organisms, such as growth rate, maximum somatic size, and investment into male and female offspring. We asked if suites of correlated traits fall into clusters defined by the Principal Components, and examined the loadings of traits included in each cluster for evidence of trade-offs that would result from developmental or energetic constraints. Further, we asked if variation for these clusters is based on genetic variation among parasite clones. Our results show that the 10 life history traits we measured are grouped into 4 independent clusters: Rate/Peak, Timing, Growth Shape, and Sex Ratio. These trait groups did not vary among parasite genotypes with the exception of Sex Ratio. We now discuss the biological relevance of the trait clusters and comment on the lack of clonal variation for these clusters except for Sex Ratio.

Rate/Peak

The Rate/Peak Principal Component indicates that the growth rate and maximal density (parasitemia) of asexual and gametocyte stages are linked into a single life history trait. It appears that faster growing infections are more capable of reaching a higher parasitemia during some fixed time. This confirms the pattern seen previously for this and other *Plasmodium* species (e.g. Mackinnon and Read 1999a; Eisen and Schall 2000), and supports the idea that rapid growth (and probable higher virulence) likely increases transmission opportunities through higher gametocytemia.

An oddity of the life history of mammalian malaria parasites is their apparent reproductive restraint; that is, although the gametocytes are the only cells that make the transition to the insect vector, few are produced in the blood (Taylor and Read 1997; Mideo and Day 2008; Pollitt et al. 2011). However, *P. mexicanum* (and other lizard malaria parasites we have examined) typically produces large numbers of gametocytes with no clear restraint, and here we again observed that infections with high asexual growth rate and peak parasitemia also had high gametocyte rate and peak. The fact that there is a positive rather than negative association between asexual and gametocyte numbers suggests a difference among infections of total energy investment rather than a difference in how that energy is allocated. Though cells that differentiate into gametocytes are no longer available to continue asexual proliferation (the classic trade-off between reproduction and somatic growth of life history theory, Gotelli 2008) the observed positive association between gametocyte and asexual parasite numbers may reflect variation in host suitability (resource availability, strength of immune response). Indeed, there is often a positive correlation between size and reproductive capacity among individuals (rather than species or clades) due to variation in their ability to obtain and assimilate resources, meaning that some individuals will have more energy to invest in both growth and reproduction (Van Noordwijk and De Jong 1986). Variation in host suitability would not be surprising because we performed our experiment using wild-caught lizards.

Timing

The Timing Principal Component includes the timing of asexual and gametocyte peak parasitemia and, to a lesser extent, patency in the blood after the day of inoculation. The relationship between asexual and gametocyte timing in reaching maximum parasitemia could indicate that asexual replication occurs for some fixed time, then switches to production of gametocytes, with the peak of gametocytes following the peak in asexual parasites on a fixed schedule. However, because most infections were still growing on the last date sampled (45/64 had maximum asexual count on last day sampled, 36/64 for gametocytes), the pattern may in part be indicative of variation in the number of days different hosts lived. Buckling et al (1997, 1999) also found that gametocytes of *P. chabaudi* are produced on schedule in infections, but heavy mortality of asexual stage parasites driven by introduction of several antimalarial drugs shifts gametocyte production earlier, exactly what life history theory predicts (Reznick and Endler 1982).

Growth Shape

The Growth Shape Principal Component describes the degree to which the growth trajectory of the infection fits an exponential curve. An exponentially growing infection results when there is a constant rate of growth (r in the exponential growth equation of $dN/dt = rN$). Deviations from exponential growth due to variation in r would occur when resources are shunted toward production of gametocytes, if the parasite experiences interference from the immune system, if resource availability varies over time, or if growth rate is adjusted for other reasons (such as gradual leveling off when a maximum

parasitemia is reached). Most infections in our study grew close to exponentially (median $R^2 = 0.86$), but despite substantial differences among infections in shape (R^2 0.31 – 0.99) and rate of increase (400-fold for asexuals, 56-fold for gametocytes), Growth Shape was independent from other life history traits, including how rapidly the infection grew.

Sex Ratio

Gametocyte sex ratio was the only trait that showed significant variation among parasite genotypes. This result agrees with a previous study on replicate single-clone infections of *P. mexicanum* (Neal and Schall 2010) as well as data for two other *Plasmodium* species (*P. falciparum* in humans and *P. chabaudi* in rodents) (Burkot et al 1984; Reece et al 2008). The relative proportion of male and female gametocytes transmitted to the insect vector should play an important role in the number of mated parasites and thus ultimate transmission success into the next vertebrate host. That is, a bias toward females would result in a larger number of zygotes produced. Sex ratio theory predicts female-biased sex ratios for single-clone infections, but posits that mixed-clone infections should produce a more balanced gametocyte sex ratio when males of different clones compete for relative local success (Düsing 1884 (translation in Edwards 2000); Fisher 1930; Hamilton 1967; Schall 2009; West 2009).

Why should gametocyte sex ratio differ among single-clone infections and why should this variation have a genetic basis? We first suspected that other life history traits might constrain allocation of cells to males and females, but this proved incorrect. It is possible that sex ratio is linked to male gametocyte fecundity (Neal, 2011), but this would

beg the question of why there is genetic variation for such an important life history trait as male fecundity.

Variation among genotypes and environmental influences

Life history traits are assumed to be under strong selective pressure, yet heritable variation typically remains substantial for multicellular species (Mousseau and Roff 1987; Stearns 1992). Data for malaria parasites are still surprisingly scant, but genetic variation for life history traits has been reported for species including *P. falciparum*, the most important human malaria parasite, and *P. chabaudi* in laboratory experimental infections (reviewed by Reece et al 2009). A previous study on *P. mexicanum* found that donor lizard whose blood was used to initiate replicate experimental infections accounted in part for variation in life history traits, but a confounding factor was lack of information on the clonal diversity of these infections (Eisen and Schall 2000). Using only single-clone infections, we found no evidence of genetic variation for the life history traits summarized as Rate/Peak, Timing, and Growth Shape, which argues that the effect reported by Eisen and Schall (2000) was indeed driven by interaction among clones in mixed-genotype infections. Vardo-Zalik and Schall (2009) found that clonal diversity was associated with shifts in life history traits of *P. mexicanum*.

It is possible that the lizards used as the source of blood to initiate single-clone infections may have harbored closely related parasites with little genetic variation, and this led to the lack of detected genetic variation for life history traits. Conflicting with this notion, Sex Ratio shows a clone effect despite rather little variation in sex ratio among infections (36 – 58% males). Additionally, these infections were taken from 5

unconnected sites, and a previous study showed that two of these sites' parasite populations are genetically differentiated from one another (Fricke et al 2010).

What, then, accounts for the substantial variation observed in life history traits (Table 8)? Although we attempted to reduce possible environmental (host) variation by selecting only adult male lizards from a very restricted area at the field site, the hosts most likely differ physiologically (erythrocyte density, blood haemoglobin concentration, serum glucose levels, and immune competency). A high degree of environmental variation could make detection of a genotype effect less feasible. Previous studies have found variation in many physiological traits in these lizards (Schall et al 1982; Schall 1990; Dunlap and Schall 1995), which may account for the variation in parasite life history traits observed. An intriguing question remains: are life history differences seen among hosts an adaptive response by the parasite to different environmental conditions, an adaptive interaction by both parasite and host, or simply an imperfect response of the parasite to environmental challenges?

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References

- Al-Olayan EM, Williams GT, Hurd H (2002) Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *Int. J. Parasitol.* 32: 1133-1143.
- Anderson TJC, Williams JT, Nair S et al (2010) Inferred relatedness and heritability in malaria parasites. *Proc. R. Soc. Lond. B* 277: 2531-2540.
- Barclay VC, Sim D, Chan BHK et al (2012) The evolutionary consequences of blood-stage vaccination on the rodent malaria *Plasmodium chabaudi*. *PLoS Biology* 10: e1001368.
- Bromwich CR and Schall JJ (1986) Infection dynamics of *Plasmodium mexicanum*, a malarial parasite of lizards. *Ecology* 67: 1227-1235.
- Buckling AG, Crooks L, Read AF (1999) *Plasmodium chabaudi*: effect of antimalarial drugs on gametocytogenesis. *Exp. Parasitol.* 93: 45-54.
- Buckling AG, Taylor LH, Carlton JM et al (1997) Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proc. R. Soc. Lond. B* 264: 553-559.
- Burkot TR, Williams JL, Schneider I (1984). Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. R. Soc. Trop. Med. Hyg.* 78: 339-341.
- Charnov EL (1993) Life history invariants: some explorations of symmetry in evolutionary ecology. Oxford University Press, New York.
- de Roode JC, Helinski MEH, Anwar MA et al (2005) Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Amer. Nat.* 166: 531-542.
- Dunlap KD, Schall JJ (1995) Hormonal alterations and reproductive inhibition in male fence lizards (*Sceloporus occidentalis*) infected with the malaria parasite *Plasmodium mexicanum*. *Physiol. Zool.* 68: 608-621.
- Edwards AWF (2000) Carl Düsing (1884) on the regulation of the sex-ratio. *Theor. Pop. Biol.* 58 255-257.
- Eisen RJ (2000) Variation in life-history traits of *Plasmodium mexicanum*, a malaria parasite infecting western fence lizards: a longitudinal study. *Can. J. Zool.* 78: 1230-1237.

Eisen RJ, Schall JJ (2000) Life history of a malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *Proc. R. Soc. Lond. B* 267: 793-799.

Fisher RA (1930) The genetical theory of natural selection. Dover Publications, New York.

Ford AM, Schall JJ (2011) Relative clonal proportions over time in mixed-genotype infections of the lizard malaria parasite *Plasmodium mexicanum*. *Int. J. Parasitol.* 41: 731-738.

Fricke JM, Vardo-Zalik AM, Schall JJ (2010) Geographic genetic differentiation of a malaria parasite, *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*. *J. Parasitol* 96: 308-313.

Gotelli NJ (2008) A primer of ecology. Sinauer Associates, Sunderland MA.

Gotelli NJ, Ellison AM (2004) A primer of ecological statistics. Sinauer Associates, Sunderland MA.

Graves PM, Carter R, McNeill KM (1984) Gametocyte production in cloned lines of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* 33: 1045-1050.

Guilbride DL, Guilbride PDL, Gawlinski P (2012) Malaria's deadly secret: a skin stage. *Trends Parasitol.* 4: 142-150.

Hamilton WD (1967) Extraordinary sex ratios. *Science* 156: 477-488.

Joliffe IT (2002) Principal component analysis: Second edn. Springer, New York.

Mackinnon MJ, Read AF (1999a) Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* 53: 689-703.

Mackinnon MJ, Read AF (1999b) Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. *Proc. R. Soc. Lond. B*: 266: 741-748.

Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life history traits and host switches. *Mol. Phylogenet. Evol.* 47: 261-273.

Mideo N, Day T (2008) On the evolution of reproductive restraint in malaria. *Proc. R. Soc. Lond. B*. 275: 1217-1224

Mousseau TA, Roff DA (1987) Natural selection and the heritability of fitness components. *Heredity* 59: 181-197.

- Neal AT (2011) Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*. Parasitology 138: 1203-1210.
- Neal AT, Schall JJ (2010) Gametocyte sex ratio in single-clone infections of the malaria parasite *Plasmodium mexicanum*. Parasitology 137: 1851-1859.
- Osgood SM, Eisen RJ, Schall JJ (2002) Gametocyte sex ratio of a malaria parasite: experimental test of heritability. J. Parasitol. 88: 494-498.
- Osgood SM, Wargo A, Eisen RJ et al (2003) Manipulation of host's testosterone does not affect gametocyte sex ratio of a malaria parasite. J. Parasitol. 89: 190-192.
- Osgood SM, Schall JJ (2004) Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. Parasitology 128: 23-29.
- Paul RE, Day KP (1998) Mating patterns of *Plasmodium falciparum*. Parasitol. Today 14: 197-202.
- Perkins SL, Osgood SM, Schall JJ (1998) Use of PCR for detection of subpatent infections of lizard malaria: implications for epizootiology. Molec. Ecol. 7: 1587-1590.
- Pollitt LC, Mideo N, Drew DR, et al (2011) Competition and evolution of reproductive restraint in malaria parasites. Am. Nat. 177: 358-367.
- Read, AF, Day KP (1992) The genetic structure of malaria parasite populations. Parasitol. Today 8: 239-242.
- Reece SE, Drew DR, Gardner, A (2008) Sex ratio adjustment and kin discrimination in malaria parasites. Nature 453: 609-614.
- Reece SE, Ramiro RS, Nussey DH (2009) Plastic parasites: sophisticated strategies for survival and reproduction. Evo. Appl. 1: 11-23.
- Reznick D, Endler JA (1982) The impact of predation on life history evolution in the Trinidadian guppies (*Poecilia reticulata*). Evolution 36: 160-177.
- Schall JJ (1989) The sex ratio of *Plasmodium* gametocytes. Parasitology 98: 343-350.
- Schall JJ (1990) Virulence of lizard malaria: the evolutionary ecology of an ancient parasite-host association. Parasitology 100: S35-S52.
- Schall JJ (1996) Malaria parasites of lizards: diversity and ecology. Adv. Parasitol. 37: 255-333.

- Schall JJ (2009) Do malaria parasites follow the algebra of sex ratio theory? *Trends Parasitol.* 25: 120-123.
- Schall JJ, Bennett AF, Putnam RW (1982) Lizards infected with malaria: physiological and behavioral consequences. *Science* 217: 1057-1058.
- Schall JJ and Marghoob AB (1995) Prevalence of a malarial parasite over time and space: *Plasmodium mexicanum* in its vertebrate host, the western fence lizard, *Sceloporus occidentalis*. *J. Anim. Ecol.* 64: 177-185.
- Schall JJ and St. Denis KM (2013) Microsatellite loci over a thirty-three year period for a malaria parasite (*Plasmodium mexicanum*): bottlenecks in effective population size and effect on allele frequencies. *Parasitology* 140: 21-28.
- Schall JJ and Vardo AM (2007) Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Molec. Ecol. Notes* 7: 227-229.
- Stearns SC (1992) The evolution of life histories. Oxford University Press, New York.
- Taylor LH, Read AF (1997) Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today* 13: 135-140.
- Taylor LH, Walliker D, Read AF (1997) Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. *Proc. R. Soc. Lond. B*: 264: 927-935.
- Van Noordwijk AJ, De Jong G (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. *Amer. Nat.* 128: 137-142.
- Vardo AM, Schall JJ (2007) Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Mol. Ecol.* 16: 2712-2720.
- Vardo-Zalik AM, Schall JJ (2008) Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 135: 1363-1372.
- Vardo-Zalik AM, Schall JJ (2009) Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* 90: 529-536.
- West SA (2009) Sex allocation. Princeton University Press, Princeton.

CHAPTER 7: Local mate competition and transmission bottlenecks: a new model for understanding malaria parasite and other sex ratios

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Highlights

- Local Mate Competition assumes females increase transmission to the next generation
- Malaria female production and transmission success may not be linearly related
- We model the effect of a non-linear female-transmission relationship on sex ratio
- Decreasing fitness returns on female production favors less investment in females
- More offspring per patch also favors more equal investment in males and females

Abstract

The local mate competition model from sex ratio theory predicts female-biased sex ratios in populations that are highly subdivided during mating, and is thought to accord well with the population structure of malaria parasites. However, the selective advantage of female-biased sex ratios comes from the resulting increase in total reproductive output, an advantage the transmission biology of malaria parasite likely reduces. We develop a mathematical model to determine how bottlenecks in transmission that cause diminishing fitness returns from female production affect sex ratio evolution. We develop four variations of this model that incorporate whether or not parasite clones have the ability to detect others that occupy the same host and whether or not the number of clones affects the total mating population size. Our model indicates that transmission bottlenecks favor less female-biased sex ratios than those predicted

under LMC. This effect is particularly pronounced if clones have no information about the presence of coexisting clones and the number of mating individuals per patch is fixed. The model extends our understanding of malaria parasite sex ratios in three main ways. First, it identifies inconsistencies between the theoretical predictions and the data presented in a previous study, and proposes revised predictions that are more consistent with underlying biology of the parasite. Second, it accounts for the positive association between parasite density and sex ratio observed within and between some species. Third, it predicts a relationship between mortality rates in the vector and sex ratios, which appears to be supported by the little existing data we have. While the inspiration for this model came from malaria parasites, it should apply to any system in which per capita dispersal success diminishes with increasing numbers of females in a patch.

1. Introduction

Evolutionary models rest on their underlying assumptions, with some models more robust than others to deviations from those assumptions. Stressing a model by altering its assumptions is often very informative, and can lead to intriguing and unexpected insights. We focus on one example from sex ratio theory, a prominent focus in evolutionary biology that makes quantitative predictions about how natural selection will shape the ratio of males to females in populations (West 2009). The early models in sex ratio theory, which were conceived by Düsing (1884, translation in Edwards 2000) and popularized by Fisher (1930), considered large, randomly mating populations. They concluded that equal investment in males and females would be favored because parents producing the less common sex would receive a fitness advantage due to the greater

mating success of their offspring. The first major extension of the theory came from Hamilton (1967), who showed that modifying certain key assumptions of the Fisherian model could favor strongly biased sex ratios. For example, if, during mating, a population is highly subdivided into patches containing the offspring of one or a few mothers and these offspring compete with one another for breeding opportunities, then female-biased sex ratios can be favored by the resulting increase in the reproductive value of the offspring or, equivalently, in the total number of grand-offspring produced.

This model, termed Local Mate Competition (LMC), has received support from studies of a wide range of arthropods whose population structure matches this model well (reviewed by West 2009). LMC has also been tested for malaria parasites (*Plasmodium* and related genera sensu Martinsen et al. 2008) with more limited success (reviewed by Schall 2009). Deviations from the predictions of LMC have often been attributed to adaptive sex ratio adjustments to compensate for limited male fecundity or mating group size within a patch (Fertility Insurance, Shutler and Read 1998; West et al. 2001; 2002). Here we consider another possibility—that the life cycle of malaria parasites deviates from the standard assumptions of LMC in a way that may alter sex ratio predictions—and we determine what sex ratios would be favored if such deviations were accounted for.

Malaria parasites have a two-host life cycle (Fig. 19). Asexual replication in a vertebrate host culminates in the production of male and female sexual cells, the gametocytes, which are the only parasite stage to survive transmission to the second host, a blood-feeding insect vector. Mating takes place within minutes of entering the vector, with female gametocytes producing one female gamete and males producing up to 8 flagellated male gametes. Following mating, zygotes undergo further asexual replication

and become oocysts, with each oocyst containing 1000s of transmission stage parasites, the sporozoites (Rosenberg and Rungsiwongse 1991). The sporozoites travel to the insect's salivary glands from whence they will be transmitted the next time the insect feeds.

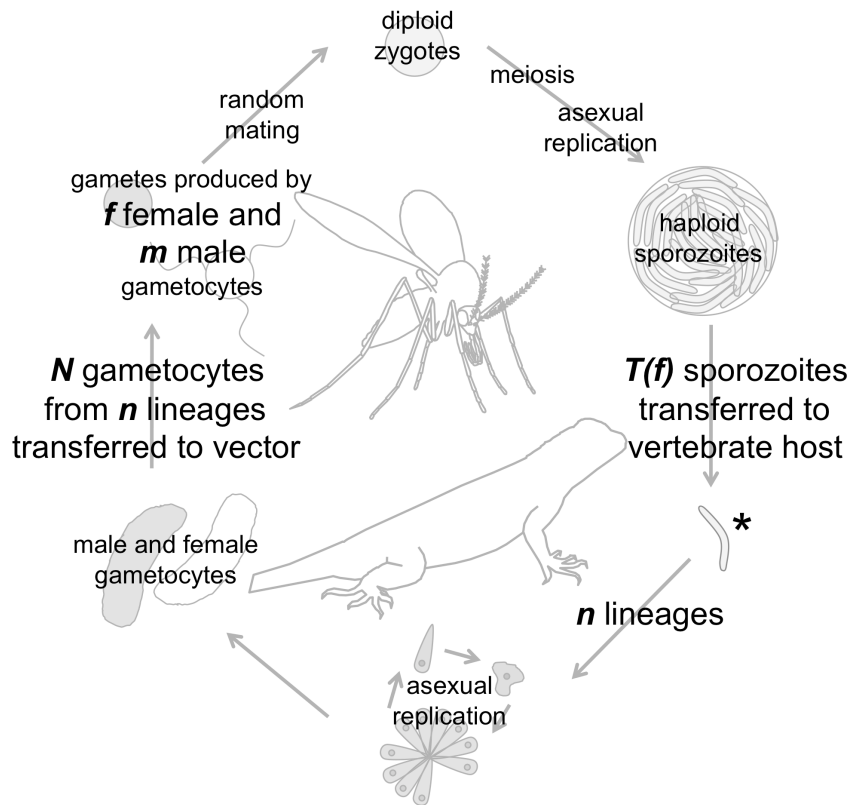


Figure 19: Starting at the asterisk (bottom right), a vertebrate host harbors n clonal parasite lineages one of which is mutant. These lineages each reproduce asexually and ultimately produce male and female sexual cells, termed gametocytes. Transmission through a blood meal provides f female and m male gametocytes to the vector, where $N = f + m$ is regarded as a function of n . Gamete production and random mating produce diploid zygotes in numbers proportional to f and these eventually produce a large number of haploid sporozoites. These are transmitted to a new vertebrate host in numbers $T(f)$ written as function of f but with diminishing returns (Fig. 20). These start the cycle again. This life cycle is common to all malaria parasites, though the specific hosts pictured are those of the lizard malaria parasite *Plasmodium mexicanum*.

The division of malaria parasites in separate insect vectors during mating coupled with the presence of many parasites deriving from only one or a few clonal lines (we

refer to these as clones or lineages; they are the result of asexual replication in both insect and vertebrate host; Paul and Day 1998, Read and Day 1992) apparently match the population structure assumed by LMC well (Read et al. 1992). However, despite the similarities of the malaria life cycle to the standard LMC model, the advantage of producing a female-biased sex ratio could be reduced if the number of female gametocytes ingested isn't linearly related to ultimate transmission success. If increased female production does not increase patch productivity at all, no female bias is favored (Colwell 1981). Intermediate situations in which producing more female gametocytes increases transmission somewhat, but not proportionally, are also possible and are explored here. For malaria parasites, having a linear relationship between female production and transmission success would require the number of female gametocytes ingested to be directly proportional to the number of zygotes, oocysts and sporozoites produced and for the number of sporozoites produced to be directly proportional to the probability of initiating a new infection.

Several characteristics of malaria parasite transmission biology make it possible that increased female production does not linearly increase transmission success. For example, there is evidence of density-dependent mortality during zygote development (Zollner et al. 2006); even if more zygotes are formed when sex ratios are female-biased, higher mortality rates may prevent a proportional increase in oocyst and sporozoite production. Furthermore, increased sporozoite production itself may not greatly affect transmission success. Sporozoites are passed to a new vertebrate host from the salivary glands of the insect vector, which may become saturated. Also, transmission is relatively efficient even with very few sporozoites (Klein et al. 1987, Ungureanu et al. 1976). One

study found that doses of 10 to 10,000 sporozoites were all equally capable of inducing infection, with only a slight reduction in time to patency between the highest and lowest dose (12 days for 10,000, 16 – 17 days for 10; Ungureanu et al. 1976). These traits almost certainly complicate the relationship between the number of females present in the mating population and transmission success, a relationship on which the selective advantage of female-biased sex ratios depends.

Here we develop a mathematical model to explore the consequences of a non-linear female-transmission relationship on the evolutionarily stable sex ratio. We compare four variations of this model that incorporate differences in the information available to the clones and the effects of multiple co-occurring lineages on the total number of offspring in the patch. These variations are: 1) plastic/additive—clones are able to detect and respond to the presence of other lineages occupying the same patch (i.e. sex ratios display phenotypic plasticity *sensu* Schall 2009) and each additional clone increases the total mating population by a set amount (additive), 2) plastic/fixed—sex ratios are plastic but the total mating population size does not depend on the number of lineages, 3) uniform/additive—clones are not able to detect or respond to the presence of co-occurring lineages or the size of the mating population (i.e. sex ratios in all patches are uniform regardless of the number of lineages and are locally adapted to maximize fitness given the distribution of patch types in the overall population, as described in Schall 2009) and mating population size is additive, and 4) uniform/fixed—sex ratios are uniform across patch types and offspring numbers per patch are fixed. We compare the sex ratio predictions derived from this model with those of standard LMC for both individual infections and population-wide patterns, including the patterns predicted by

Read et al.'s (1995) derivation relating parasite prevalence in a host population with predicted sex ratio (see section 3.4). In sections 3.5 and 3.6, we discuss how these predictions relate to existing data on malaria parasite sex ratios and how the model may apply to other biological systems.

2. Methods

2.1 General model

The general parasite life cycle is diagrammed in Fig. 19. By analogy with the standard LMC model (Hamilton 1967), we interpret this as an infinite island model in which the patch is the insect vector, the mated females who disperse are the diploid zygotes, and their offspring are the sporozoites, which multiply and ‘mature’ into male and female gametocytes in a vertebrate host. N gametocytes are passed to the insect vector: these derive equally from the n clones that colonized the vertebrate host and mate at random to produce the next generation of zygotes.

The two transmission episodes in the Fig. 19 life cycle provide two potential “bottlenecks” and we treat each of them differently. The transmission of the sporozoites down to the vertebrate is modeled with diminishing returns using the functional form:

$$T(f) = 1 - e^{-af} \tag{1}$$

(Fig. 20) with a parameter a , which regulates the strength of the effect. This will indeed have an effect on the sex ratio dynamics as, for a particular N , a greater female bias will increase f and thereby decrease the marginal transmission rate.

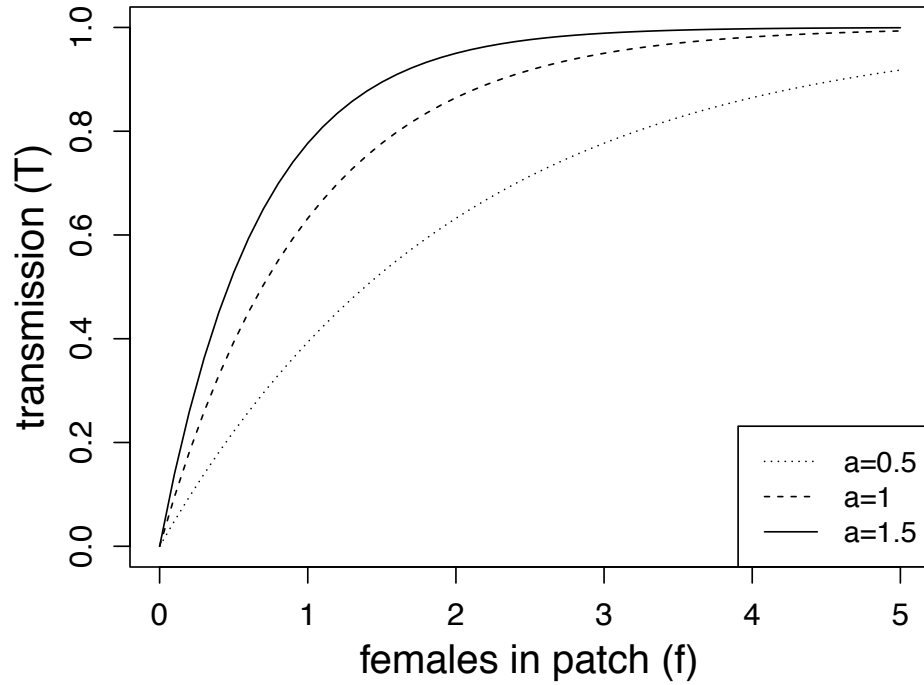


Figure 20: A plot of Eq. 3 showing the effect of a on the relationship between female production (f) and transmission success (T).

On the other hand, the transmission of the gametocytes to the vector is modeled through the effect of n on N , and we use the general form

$$N = \hat{n} \left(\frac{n}{\hat{n}} \right)^\beta \quad (2)$$

Here β is a parameter ($0 \leq \beta \leq 1$) measuring the extent to which N responds to variations in n . With $\beta = 0$ there is no response and N has the “fixed” value \hat{n} ; with $\beta = 1$ there is an “additive” response and N takes the value n . Thus in the fixed case, the N transmitted gametocytes comprise n clones of equal size but N is the number that would be transmitted in the additive case when there are \hat{n} clones.

Our analysis will keep track of the change in the number of mutant zygotes from one generation to the next, that is, over a complete cycle (Fig. 19). Before we turn to that it is worth mentioning that in a single cycle the parasite will in general encounter two

values of the lineage number n : values n_0 in the vertebrate and n_1 in the vector. Generally there is a single blood meal between the two hosts and the n -values are equal or at least highly correlated, and we will in fact work with a single n . But for clarity we observe that n_0 carries the information used by the sporozoite in determining the sex ratio (in the plastic case) whereas n_1 determines the size of f which feeds into the second bottleneck $T(f)$.

We track mutant fitness beginning with a mutant zygote and its clonal derivatives in the vertebrate. Our assumption of $n-1$ resident and 1 mutant clone (with sex ratios r and s respectively as in Taylor and Bulmer 1980) gives us an overall sex ratio of

$\frac{(n-1)r + s}{n}$ and this determines the numbers of male and female gametocytes in the

vector as:

$$\begin{aligned} m &= N \left(\frac{(n-1)r + s}{n} \right) \\ f &= N \left(\frac{(n-1)(1-r) + (1-s)}{n} \right) \end{aligned} \quad (3)$$

The mutant genetic representation in these male and female gametocytes is

$$\begin{aligned} p_M &= \frac{s}{(n-1)r + s} \\ p_F &= \frac{1-s}{(n-1)(1-r) + 1-s} \end{aligned}$$

so that after mating the overall proportion of mutant genes in the diploid zygotes will be

$$p = \frac{p_M + p_F}{2}. \quad (4)$$

For each value of n , mutant fitness can be measured as the mutant's share of sporozoites transmitted to the vertebrate:

$$w(n) = w(s, r; n) = p T(f). \quad (5)$$

2.2 Equilibrium equations.

We now formulate the equations for evolutionary equilibrium of the sex ratio for each of our four variations.

2.2.1 Variations 1 and 2: Plastic

The parasite can determine and respond to the value of n . The N -bottleneck is additive ($\beta = 1$) or fixed ($\beta = 0$). In both cases, the sex ratio equilibrium is attained at the value $r = r^*$ at which

$$\left. \frac{\partial w}{\partial s} \right|_{s=r=r^*} = 0. \quad (6)$$

2.2.2 Variations 3 and 4: Uniform

The parasite is unable to determine the value of n , and evolution responds to a suitable average value of n . We describe the distribution of n among patches with the negative binomial (B) using the parameterization given in Bliss and Fisher (1953). The two necessary parameters are μ , the mean number of lineages per patch, and k , which is inversely related to the degree of aggregation of lineages among patches. When k is high, the negative binomial closely approximates a Poisson distribution. When k is low, the variance of the distribution is high relative to its mean because lineages tend to clump together, creating more patches with many or few lineages than expected by chance (i.e. under a Poisson distribution). This makes the negative binomial useful for describing the distribution of parasites in a population of hosts because a small proportion of hosts often harbors the majority of parasites (Anderson and May 1991, Crofton 1971). In fact, many

plants and animals show significant aggregation (Bliss and Fisher 1953) so the distribution should be generally applicable. Taking B_n to be the frequency of patches of type n , the probability of a clone being in a patch with n clones is

$$Q_n = \frac{nB_n}{\sum_i iB_i} \quad (7)$$

where the sum runs over the range of possible patch types. Mutant fitness is then measured as an average taken across all patch types:

$$W = \sum_n w(n)Q_n \quad (8)$$

using the fitness function of eq. (5). For most runs we used an n -range between 1 and 1000. The high maximum n is only necessary when μ is high (e.g. for high prevalence- see section 3.4) but was used elsewhere for consistency.

The N -bottleneck is again additive ($\beta = 1$) or fixed ($\beta = 0$) and in each case the sex ratio equilibrium is attained at the value $r = r^*$ at which

$$\left. \frac{\partial W}{\partial s} \right|_{s=r=r^*} = 0. \quad (9)$$

Our equilibrium eqs. (6) and (9) cannot be solved analytically and numerical solutions for a variety of parameter combinations are presented in the results section.

2.3 Population-wide sex ratios

Some studies of malaria parasite sex ratios have compared the average population sex ratios (across multiple patches) of different populations. It may therefore be useful to know what average population sex ratio is predicted by different variations of the model.

Average population sex ratios are also helpful for comparing the predictions of different model variants because the predicted sex ratios of some variants depend on n (both plastic variants) and some do not (both uniform variants).

For both of the uniform variations of the model (2.2.2), the same sex ratio is expected in all patch types, and this will be the average population sex ratio. For both plastic variations (2.2.1), the sex ratios predicted will vary based on n , and the average sex ratio across all patches will depend on the frequency of patch types in the population. In this case, the average clone sex ratio is a weighted average of the sex ratios predicted in each patch type:

$$r^*_{pop} = \sum_n r^*_n Q_n \quad (10)$$

with Q_n given in eq. (7).

The average patch sex ratio, which does not take into account how many lineages may share a patch and is therefore more closely comparable to existing data for malaria parasites, is:

$$r^*_{patch} = \sum_n r^*_n B_n \quad (11)$$

3. Results and discussion

3.1 The $T(f)$ bottleneck and LRC

We begin with some general remarks about sex ratio theory, designed to help readers put our results in context. Our model represents a particular case of local resource competition (LRC), the idea that the sex ratio of a population should be biased

toward the sex that competes for resources less intensely with relatives (Clark 1978). In this sense, the $T(f)$ bottleneck creates competition among females for a share of the transmission success (see Appendix for mathematical support of this claim). Of course females are themselves a significant reproductive resource for males and thus LMC is an additional source of LRC acting on males (Taylor 1981, West 2009).

To better understand the interaction between LRC among males and among females, we look at two extreme cases. At one end of the spectrum we have no bottleneck and transmission is proportional to production so $T(f) = f$. In this case the only source of LRC is the standard LMC among males (Hamilton 1967). At the other extreme is the complete bottleneck case in which $T(f)$ is independent of f and transmission is fixed. In this case males and females are faced with exactly the same level of local competition and the patch behaves like a finite random mating population for which the sex ratio is known to be unbiased (Fisher 1930). Our model lies between these two extremes; $T(f)$ behaves like f for small f and like a constant for large f (Table 9).

Table 9: The strength of the $T(f)$ bottleneck. Tabulated values of r^* belong to the plastic additive model. Variables are defined in section 2.

	fitness $w(n)$ (eq. 5)	ES sex ratio ($n = 4$)
no bottleneck pure LMC on males	$w(n) = pf$	$r^* = \frac{n-1}{2n} = 0.375$
partial bottleneck (Fig 19) LMC on males, partial LRC on females	$w(n) = pT(f)$	$r^* = 0.440$
complete bottleneck Male LMC and female LRC balanced	$w(n) = p$	$r^* = 0.500$

The additive variations of our model also have ties to the Trivers-Willard hypothesis (T-W), which proposes that progeny sex ratios should be shifted in response

to conditions (environmental or parental) that alter the relative reproductive value of male vs. female offspring (Trivers and Willard 1973). In our model an increase in n decreases per capita transmission success for both sexes (an effect of crowding), but the effect of this can be alleviated by a reduction in the number of female offspring leading to an increase in fitness per daughter (see Appendix 1). This provides an example of the interaction between LRC and T-W.

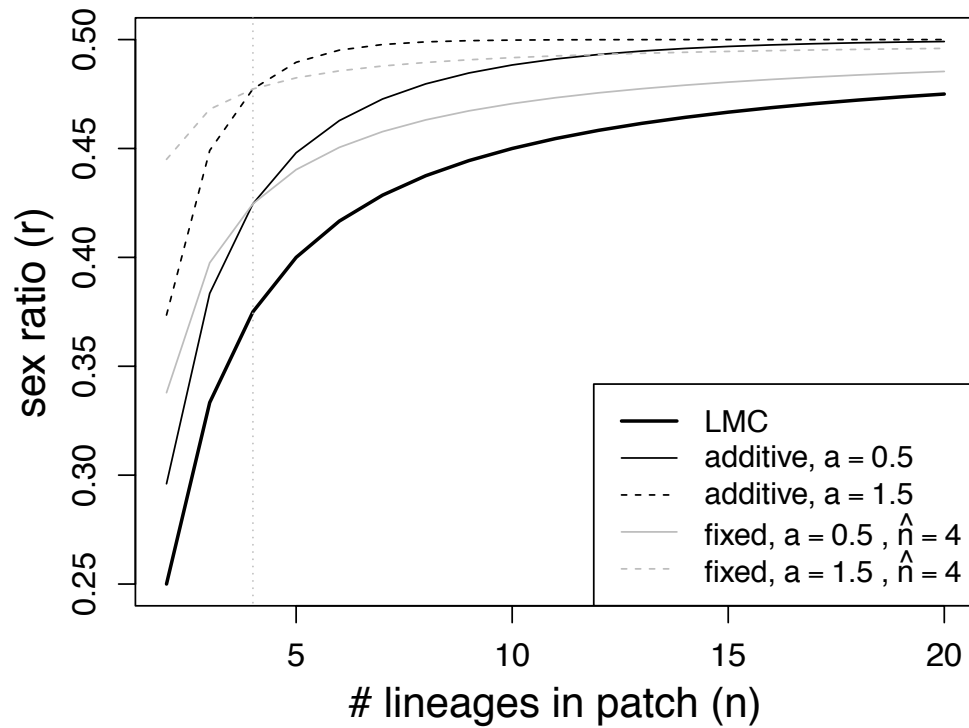


Figure 21: Model results showing the sex ratios predicted for patches with different numbers of lineages (n) under different variations and parameterizations of the model (see legend). The vertical dashed line shows $n = \hat{n} = 4$. Only the first two model variations are shown here (plastic/additive and plastic/fixed) because the predictions of the uniform model variations do not vary with n .

3.2 Sex ratios among patches

When mothers are able to detect and respond to the number of lineages present in a patch, the unbeatable sex ratios identified by our model are higher (less female-biased) than those predicted by LMC for all values of n and a we tested (Fig. 21). This result is

consistent with our intuition. The concave-down shape of the bottleneck $T(f)$ gives a higher female marginal return when fewer females are produced, thus decreasing the female-bias of the sex ratio. This effect is more pronounced for higher a (Fig. 21).

Comparing the plastic/fixed and plastic/additive cases, sex ratios in the additive case are lower when $n < \hat{n}$ and higher when $n > \hat{n}$ (Fig. 21). This result is also consistent with our expectation. The number of females in the patch at equilibrium is represented by $f = n(1 - r^*)$ (eqs. (2) and (3)) in the additive case ($\beta=1$) and by $f = \hat{n}(1 - r^*)$ in the fixed case ($\beta=0$). When $n > \hat{n}$, the additive f is the larger of the two, and therefore more influenced by the bottleneck, favoring a less female-biased sex ratio.

Overall, the relationship between the number of lineages in a patch and the patch sex ratio predicted by our model shows a similar trend to that predicted by LMC, though the specific sex ratios predicted may be substantially higher depending on the strength of the vector-to-vertebrate bottleneck.

3.3 Population-wide sex ratios

Exploring the effect of transmission bottlenecks on the population-wide sex ratio (i.e. average across all clones or patches) is useful for a number of reasons. First, if mothers are unable to detect or respond to the number of co-occurring lineages in their patches, they should produce a sex ratio that is adapted to the distribution of patch types in the population. In this case, infection and population sex ratios would be the same. Second, researchers may not have the ability to assess the numbers of lineages in individual patches but may have information about average population sex ratios and numbers of occupied patches (see section 3.4 for an example). Third, population-wide

sex ratios provide a useful way of comparing the predictions of the uniform vs. plastic variations of our models.

For all four variations of our model, sex ratios generally increase with increasing bottleneck strength (i.e. higher a) and are higher than the predictions of LMC (Fig. 22). There are two exceptions, both relating to the uniform/additive case: first, our model predicts sex ratios that are slightly lower than the corresponding LMC model under some population structures (μ and k) and second, it shows a very slight decrease in r^* over some ranges of a (e.g. see Fig. 22 when $\mu = 5$ and $k = 0.5$: the black dotted LMC line is perfectly horizontal, while the black solid model line dips slightly). We are at a loss to explain this behavior, though we note that both deviations from the general pattern are slight, occur for only a limited range of parameter values, and would be indistinguishable in a biological system.

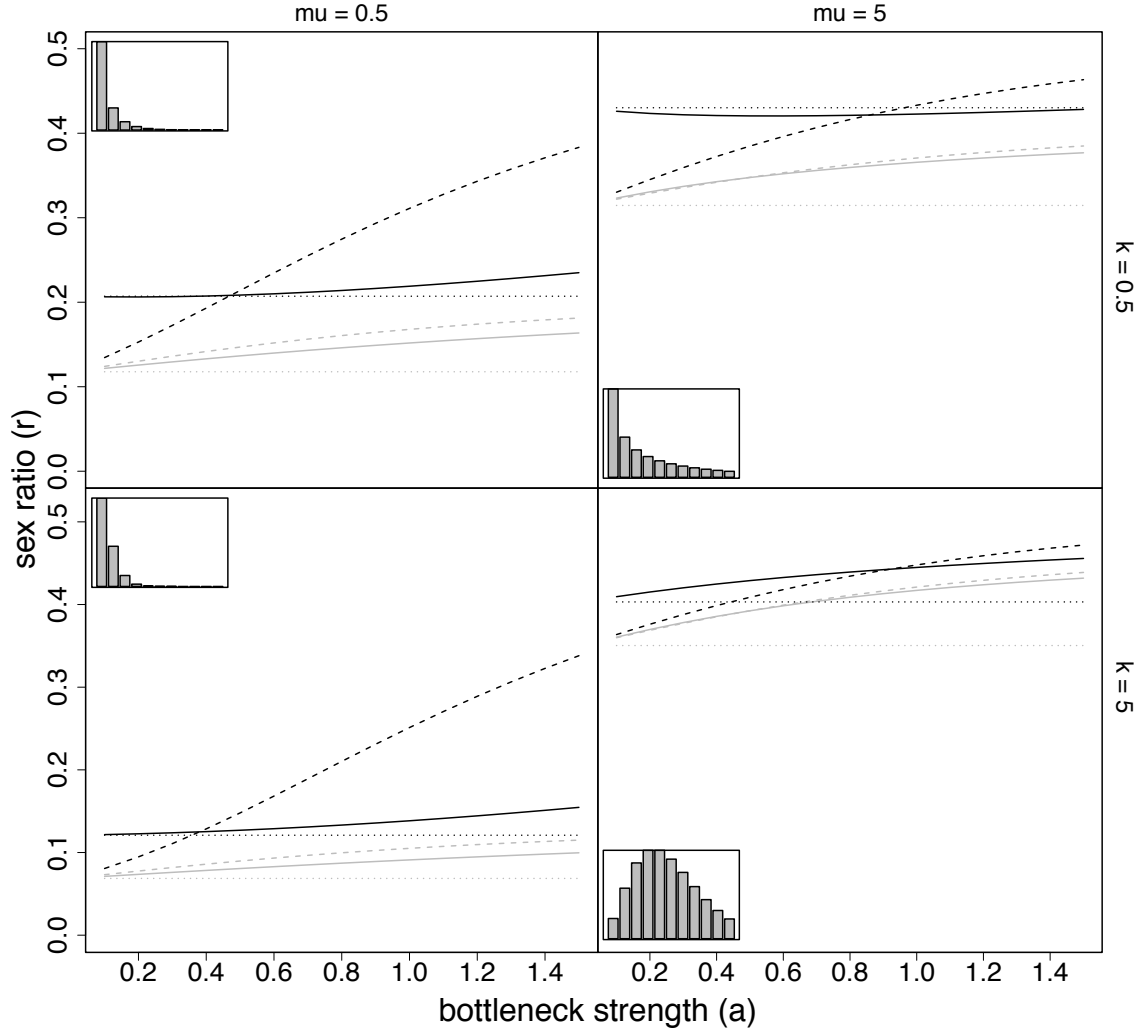


Figure 22: The effect of bottleneck strength (a) on the average population sex ratio (proportion male, r^*) for different population structures (plots) and model variations (line types). μ (μ) is the average number of lineages per patch and k is inversely related to the degree of aggregation. The small graph inset in each panel shows proportion of infections (y-axis) with each number of lineages (x-axis, ranging from 0 to 20) based on μ and k . The line types correspond to variations of the model (and match those used in Fig. 23, which provides a legend) as follows: black, solid lines are uniform/additive; black, large-dashed lines are uniform/fixed; black, small-dashed lines are LMC uniform/additive; gray solid lines are plastic/additive; gray large-dashed lines are plastic/fixed; gray small-dashed lines are LMC plastic/additive, LMC plastic/fixed and LMC uniform/fixed (all give the same prediction). All sex ratios shown for plastic model variations are averaged over patches; sex ratios averaged over all clones show the same shape relative to a but are higher. Results are from a run of the model with $\hat{n} = 4$.

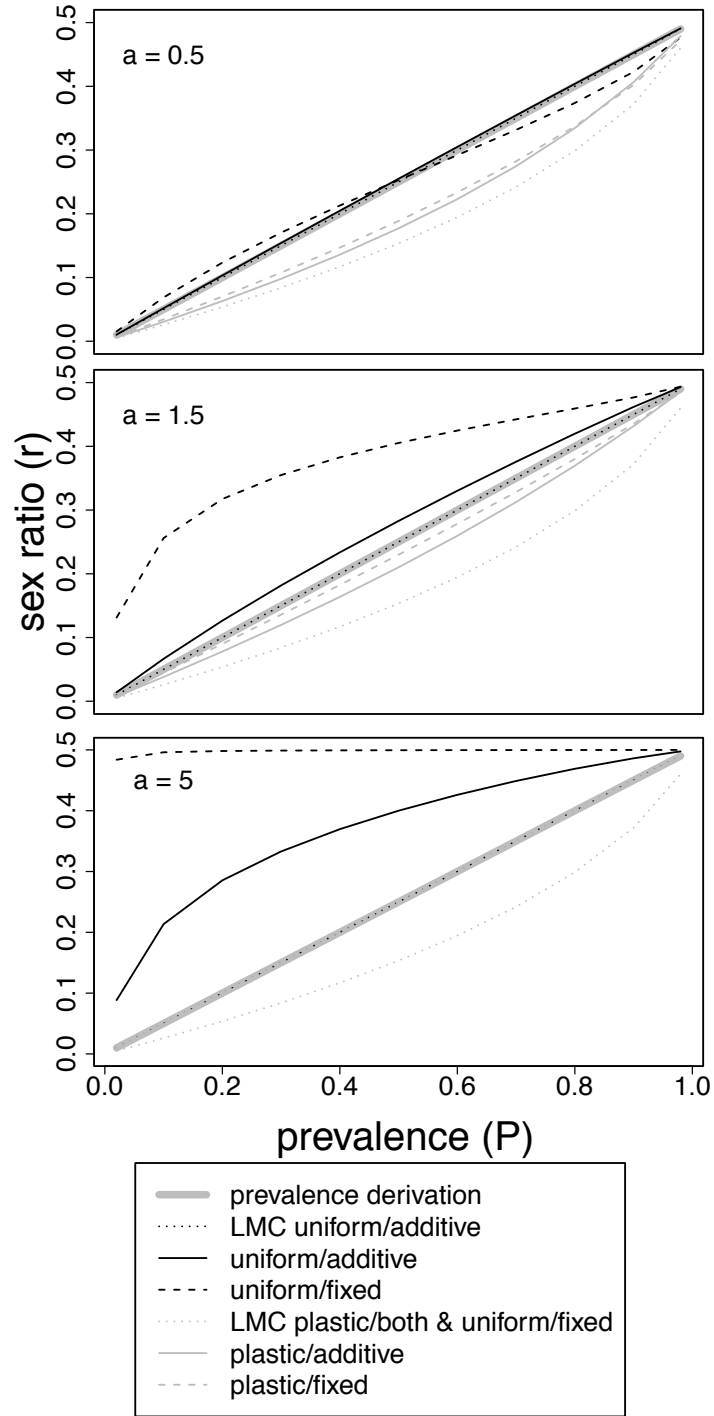


Figure 23: The predicted relationship between sex ratio (r^*) and the prevalence of a parasite in a host population (i.e. proportion of hosts infected, P). The relationship between P , μ , and k is given in section 3.4 of the text. Model variations (line types) are summarized in the legend, which also corresponds to Fig. 22. Results shown are from a run with $\hat{n} = 4$ and $k = 1$. Sex ratios from the plastic model variations are averaged over all patches. The plastic/additive and plastic/fixed model predictions are hidden by the prevalence derivation in the bottom panel.

While it is true that predicted sex ratios generally increase with increasing bottleneck strength, some variations of our model are more sensitive to these shifts in a than others. The uniform/fixed case appears to be the most sensitive to small changes in a (Fig. 22). Population sex ratios also increase with increasing μ , as in LMC, and are bounded by 0 and 0.5. Sex ratios appear to decrease slightly with increasing k , though overall predicted sex ratios are relatively insensitive to increases in k over multiple orders of magnitude, especially for lower μ (Fig. 22). The relationship between sex ratio and both μ and k is likely due to their effect on the overall population structure. As can be seen in the inset plots in Fig. 22, increasing both μ and k increases the proportion of infections that have multiple clones, thereby favoring less-biased sex ratios (as under standard LMC). It may also be worth noting that without the vector-to-vertebrate transmission bottleneck included in the model, two different sex ratios are predicted depending on the other model assumptions. While only the plastic/additive assumptions are actually consistent with standard LMC, it is worthwhile to compare sex ratios of our model with those predicted in the absence of the bottleneck (which we refer to collectively as the LMC variations). In the absence of a bottleneck, the sex ratios predicted under the uniform/fixed assumptions are lower than those predicted under the uniform/additive assumptions (Fig. 22, 23). Both plastic variations of the LMC model predict the same sex ratios, which interestingly match the uniform/additive sex ratios if averaged over all clones and match the uniform/fixed sex ratios if averaged over all patches (Fig. 22, 23).

3.4 Transmission bottlenecks, prevalence and malaria parasite sex ratios

Empirical studies of malaria parasite sex ratios in natural populations often lack developed methods for measuring the number of parasite clones present in individual infections (e.g. genetic testing; Schall 2009). Some researchers have instead used the proportion of hosts infected (also referred to as parasite prevalence, P) to estimate the average number of clones per infection in a population (Read et al. 1995, Shutler et al. 1995). If clones are distributed among hosts following a negative binomial distribution, a relatively straightforward relationship exists between prevalence and the mean number of clones per infection: $P = 1 - (1 + \mu/k)^{-k}$ (Anderson and May 1991). This expression can be rearranged to give the mean number of clones per infection based on prevalence, provided a reasonable estimate of k is available. Using this strategy, Read et al. (1995) derive an expression for predicting sex ratio directly from prevalence data (hereafter we refer to it as the “prevalence derivation” of LMC). We plotted the predictions of our model against P (using $\mu = k [(1 - P)^{-(1/k)} - 1]$ and $k = 1$; Read et al. 1995) to provide a useful framework for comparing the predictions of our model and the prevalence derivation as well as to illustrate the predicted population-wide effects of our model in a form more easily compared with existing empirical data (Fig. 23).

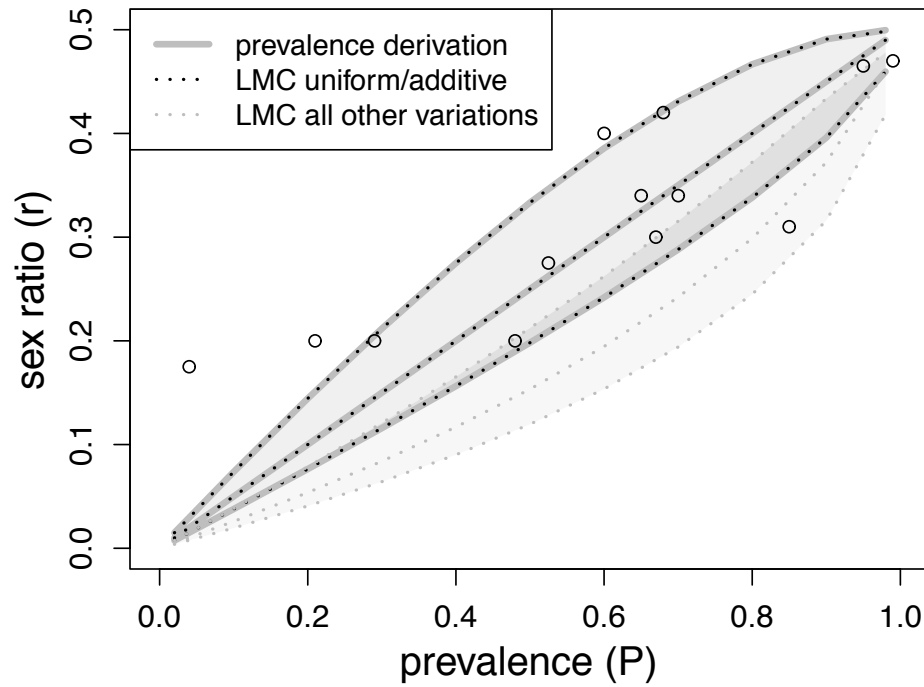


Figure 24: Comparison of data taken from Read et al. 1995 (points) with model predictions from the same paper ('prevalence derivation') and different variations of LMC. As in Read et al. 1995, three lines are provided for each model which correspond to $k = 0.5$ (top), $k = 1$ (middle) and $k = 2$ (bottom). The area encompassed by these lines is shaded to highlight the area of the graph that is consistent with each model given the range in k assumed by Read et al. 1995. Note that male fecundity sets a minimum on sex ratio predictions that is not included here but is likely important, especially for infections at low prevalence sites.

Read et al. (1995) present data on the average sex ratios for natural populations of malaria parasites in the genus *Leucocytozoon* (and one population of *Plasmodium falciparum*) that appear to match their predictions well. However, if sex ratios are averaged over all patches (which they are), the prevalence derivation's predictions match the predictions we obtained for LMC only in the uniform/additive case (Fig. 23, 24)¹. We believe that the assumptions of the uniform/additive case are unlikely to be accurate for

¹ Note that the prevalence derivation matches the predictions of our plastic/additive model variation (which incorporates assumptions consistent with the original LMC) if sex ratios are averaged over all clones. Our model is therefore consistent with their derivation, but highlights the conflict between their derivation and the data presented.

malaria parasites. Sex ratio data from multiple species suggest that malaria parasites adjust their sex ratio relative to the number of clones present (i.e. they are not uniform; e.g. *P. chabaudi*, Reece et al. 2008; *P. mexicanum*, Neal and Schall 2014b; *P. falciparum*, Sowunmi et al. 2009b), and while there is evidence that infections with more lineages produce more sexual cells (Taylor et al. 1997), this effect is not always strong (i.e. they are not additive; Vardo-Zalik 2009) or indeed observed for all species (Read and Taylor 2001). If we assume that either of these assumptions (uniform sex ratios or additive parasite numbers) is inaccurate, the LMC model does not account for the data presented in Read et al. (1995) as well (Fig. 24, “LMC all other variations”). However, the sex ratios predicted by both plastic variations of our model produce sex ratio predictions very similar to the prevalence derivation, especially when bottlenecks are strong (Fig. 23). Our model is therefore as capable as the prevalence derivation of explaining the observed relationship between prevalence and sex ratio, but our model may more closely approximate the underlying selective forces. Our model may also account for the sex ratios observed for a similar study in avian *Haemoproteus* in which observed sex ratios were uniformly just below 0.5 proportion male and not related to prevalence (Shutler et al. 1995). Such a pattern is consistent with our model, particularly if the parasite is unable to detect and respond to cues in its host or if bottlenecks are particularly strong (e.g. ‘uniform/fixed’, $a = 5$, Fig. 23).

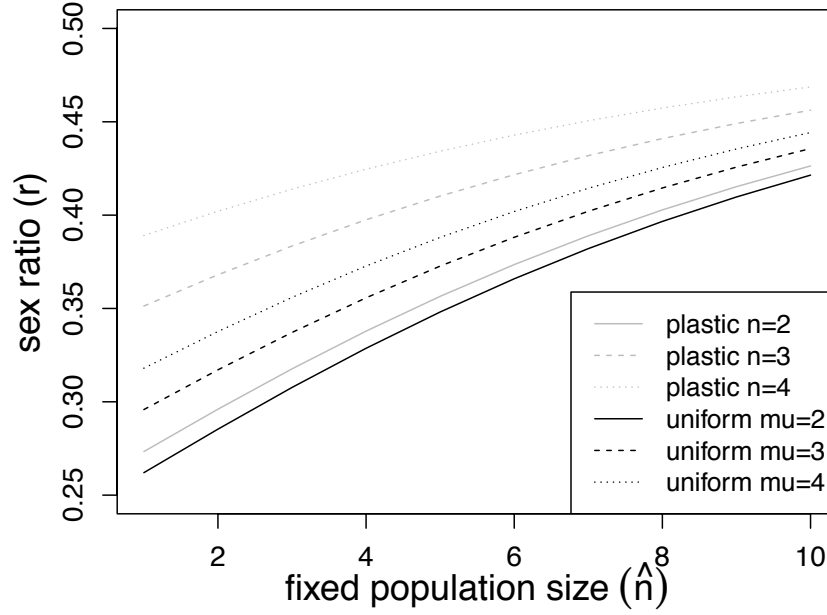


Figure 25: Predicted relationship between fixed patch size (\hat{n}) and sex ratio (r^*). Only fixed model variations are shown because \hat{n} cancels out in additive models (see eq. (2)). For the plastic/fixed variation, predicted sex ratios are for 3 different values of n ($n = 2, 3, 4$). For the uniform variation, predicted sex ratios are for three different populations with different mean number of clones per infection ($\mu = 2, 3, 4$) and $k = 0.5$.

3.5 Comparison of model with other existing data

In addition to explaining patterns in existing data on prevalence and sex ratio with more realistic assumptions than the prevalence derivation, transmission bottlenecks may also account for some previously unexplained patterns of malaria parasite sex ratios. For example, a positive relationship is sometimes observed between parasite density and sex ratio (e.g. *Plasmodium 'tropicum'*: Pickering et al. 2000; *P. mexicanum*: Neal and Schall 2014b, Schall 2000; *Plasmodium chabaudi*: Taylor 1997), which, at least for *P. mexicanum* and *P. chabaudi*, cannot be attributed to a link between the number of clones in an infection and gametocyte density (Neal and Schall 2014b, Taylor 1997). Our model shows that if patch size is fixed, increases in \hat{n} increase r^* (Fig. 25). In other words, if gametocyte density varies but does not depend on the number of clones in an infection

(i.e. it is fixed by some other variable like immune competency or resource availability), infections with higher densities of gametocytes are predicted to produce higher sex ratios. Models currently applied to malaria parasite sex ratios cannot account for a positive relationship between gametocyte density and sex ratio in the absence of a link between gametocyte density and clonal diversity.

Even though this positive relationship has been observed among infections for relatively few species (perhaps because few species have high enough average gametocyte densities to overcome the selective force of Fertility Insurance, which favors a negative relationship), the trend may also exist among species: *Plasmodium falciparum* infections typically have no more than 5% of erythrocytes infected (Mackinnon and Read 2004) and have relatively low sex ratios, generally between 10 and 20% (Read et al. 1992, Robert et al. 1996, Sowunmi et al. 2009a and b). *Plasmodium chabaudi* generally has a higher rate of infected erythrocytes, perhaps 5-30% (Mackinnon and Read 2004), and somewhat higher sex ratios (15 – 45% [Reece et al. 2008; 2003]). *Plasmodium mexicanum* infections can have up to 90% of erythrocytes infected (Vardo-Zalik and Schall 2009), sometimes with predominantly gametocytes, and has average sex ratios around 40 – 45% male (Neal 2011, Neal and Schall 2014a, 2014b, 2010, Osgood and Schall 2004, Osgood et al. 2003, 2002). More detailed data from additional species would help determine whether this pattern holds. Our model is the first to predict this relationship within or across species.

Additionally, a positive relationship between the strength of transmission bottlenecks and sex ratio would also support our model's predictions. Ideally, bottleneck strength would relate female production to ultimate transmission success (Fig. 20), but

unfortunately, we know of no such data for any species. There are, however, data from a limited number of species on the proportion of females that successfully form oocysts, which is at least a part of the path from production to transmission. *Plasmodium falciparum* appears to generally have a lower proportion of females fail to reach the oocyst stage than *P. chabaudi* (Vaughn 2007) and has correspondingly lower sex ratios (see previous paragraph). *Plasmodium vivax* has a similar or slightly higher failure rate compared with *P. falciparum* and the average sex ratio reported by one study (the only one we know of with detailed data) is 27% male (Zollner et al. 2006), which falls somewhere between *P. falciparum* and *P. chabaudi*. These data are far from conclusive—they only measure part of the relevant bottleneck and don't control for other factors that likely influence sex ratios, like the number of clones per infection, but they are suggestive of the predicted pattern and emphasize the need for more detailed within and cross species data on development in the vector and transmission.

Most other existing data on sex ratios in malaria parasites describe general patterns. These patterns, such as higher sex ratios in infections with more clones (Neal and Schall 2014b, Reece et al. 2008), are consistent with LMC whether or not transmission bottlenecks are accounted for, and are therefore not specific enough to distinguish between the two models. Data suggesting sex ratio adjustments in response to low fecundity or small mating groups are also consistent with our model. Fertility Insurance predicts an increased investment in males under certain conditions, and transmission bottlenecks would not disrupt this trend.

3.6 Extensions

Even though our focus has been on malaria parasites, our goal was to develop a model that could apply generally to different organisms. While ‘transmission bottlenecks’ sound particularly suited for a host-parasite system, by transmission we simply mean the transmission of genes from one generation to the next. Organisms could experience bottlenecks due to many different biological factors; low resource availability, predation and disease all reduce the probability that genes will be transmitted from one generation to the next. The intensity of each of these transmission blockers may also depend on the number of females in a patch, especially if females use proportionally more resources or are more attractive to predators or parasites.

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Appendix

The standard LMC model

We work with an infinite island model with demes occupied by a fixed number n of mated females. We assume that each female has a large number of male and female offspring and then dies. These offspring mate at random on the deme. Following this,

the males die and the mated female offspring disperse randomly to form new demes of size n . The problem is to calculate the evolutionarily stable sex ratio. This is the standard local mate competition (LMC) model and we will work with that here. A “partial dispersal” variation of this that we will not consider allows a fraction of the mated female offspring to remain on their native deme and compete with one another and with any immigrant females, for the n breeding slots.

Consider a focal mated female and suppose she has x daughters and y sons. For this standard LMC model her fitness w is completely determined, on average, by x and y : $w = w(x, y)$. Now let D be her fitness per daughter through daughters and let S be her fitness per son through sons. Then her fitness has the form:

$$w(x, y) = D(x, y)x + S(x, y)y. \quad (\text{A.1})$$

(Note: Her fitness is calculated as the number of copies of her genes projected into the future. Now any such copies will either descend through a daughter or a son, but not both, so her fitness is indeed a sum of two terms as above.)

If we let the sex ratio of her offspring be r , then with a fixed number of offspring, x and y are both functions of r , and we differentiate her fitness with respect to r :

$$\frac{dw}{dr} = \frac{\partial w}{\partial x} \frac{dx}{dr} + \frac{\partial w}{\partial y} \frac{dy}{dr}. \quad (\text{A.2})$$

where

$$\frac{\partial w}{\partial x} = \frac{\partial D}{\partial x} x + D + \frac{\partial S}{\partial x} y \quad \text{and} \quad \frac{\partial w}{\partial y} = \frac{\partial D}{\partial y} x + \frac{\partial S}{\partial y} y + S. \quad (\text{A.3})$$

Now with a fixed number K of offspring, $dy/dr = K$ and $dx/dr = -K$ and normalizing K to be 1, we can write (Taylor 1981):

$$\frac{dw}{dr} = \frac{\partial w}{\partial y} - \frac{\partial w}{\partial x} = (S - D) + \left(\frac{\partial D}{\partial y} - \frac{\partial D}{\partial x} \right) x + \left(\frac{\partial S}{\partial y} - \frac{\partial S}{\partial x} \right) y. \quad (\text{A.4})$$

The three brackets on the right display the different ways through which a change in sex ratio affects fitness. The first bracket provides the obvious effect on changes in the number of sons and daughters (e.g. an additional son adds S to her fitness). The second bracket provides the effect of an increase in the number of sons or daughters on her fitness per daughter and finally the third bracket does the same for fitness per son.

We now calculate D and S . Since all the daughters disperse, neither x nor y have any effect on fitness per daughter and D is a constant which we take to be 1. If there were partial dispersal of females, so that some daughters remain and compete with one another for spots on the natal deme, then we would expect $\partial D/\partial x$ to be negative. Turning to S , since all mating takes place on the deme, male fitness is entirely provided through the females on the deme so that:

$$S = \frac{(n-1)\hat{x} + x}{(n-1)\hat{y} + y}. \quad (\text{A.5})$$

Here we let \hat{x} and \hat{y} be the average number of female and male offspring of the other $n-1$ breeding females. Then:

$$\frac{\partial S}{\partial x} = \frac{1}{ny^*} \quad \text{and} \quad \frac{\partial S}{\partial y} = -\frac{1}{ny^*} \frac{x^*}{y^*} \quad (\text{A.6})$$

where the derivatives are evaluated at the population-wide equilibrium values x^* and y^* .

As expected, an increase in the number of daughters has a positive effect on the fitness of a son (more mating opportunities) whereas an increase in the number of sons has a negative effect on the fitness of a son (increased competition for matings). If we put these expressions into eq. (A.4) we get

$$\frac{dw}{dr} = \left(\frac{x^*}{y^*} - 1 \right) - \frac{1}{ny^*} \left(\frac{x^*}{y^*} + 1 \right) y^* \quad (\text{A.7})$$

Taking $y^* = r^*$ and $x^* = 1 - r^*$ and simplifying gives us:

$$\frac{dw}{dr} = \frac{1}{r^*} \left(\frac{n-1}{n} \right) - 2. \quad (\text{A.8})$$

Setting this to zero gives us an equilibrium at

$$r^* = \frac{n-1}{2n}. \quad (\text{A.9})$$

and this is convergence stable (Christiansen, 1991) as, from eq. (A.8) an increase in r^* will decrease dw/dr , making it negative, thereby encouraging the spread of sex ratio variants with smaller r . This is the standard diploid LMC result (Hamilton 1967, Taylor and Bulmer 1980), the female bias resulting from the competitive pressure among brothers for mates.

The effect of the bottleneck

Let $F = (n - 1)\hat{x} + x$ be the total number of female offspring born on the deme.

Under the standard LMC model, these F females all survive and disperse. The effect of the bottleneck is to let the actual number L of females who survive and disperse be somewhat less than F such that the probability of dispersal decreases with F . In fact we assume that the graph of L against F is increasing but with decreasing slope (Fig. 26).

Now the focal breeder will get her fair share x/F of the dispersing females, so that her fitness through daughters is xL/F and her fitness *per daughter* is $D = L/F$. Then:

$$\frac{\partial D}{\partial x} = \left(\frac{L'(F)}{F} - \frac{L(F)}{F^2} \right) \frac{dF}{dx} = \frac{1}{F} \left(L'(F) - \frac{L(F)}{F} \right) \quad (\text{A.10})$$

since $dF/dx = 1$. The concave-down form of the graph of L against F makes $\partial D/\partial x$ negative (Fig. 26) and from eq. (A.4) this increases dw/dr at the LMC equilibrium (eq. (A.9)), making it positive and thereby encouraging the spread of sex ratio variants with larger r leading to a less female-biased sex ratio.

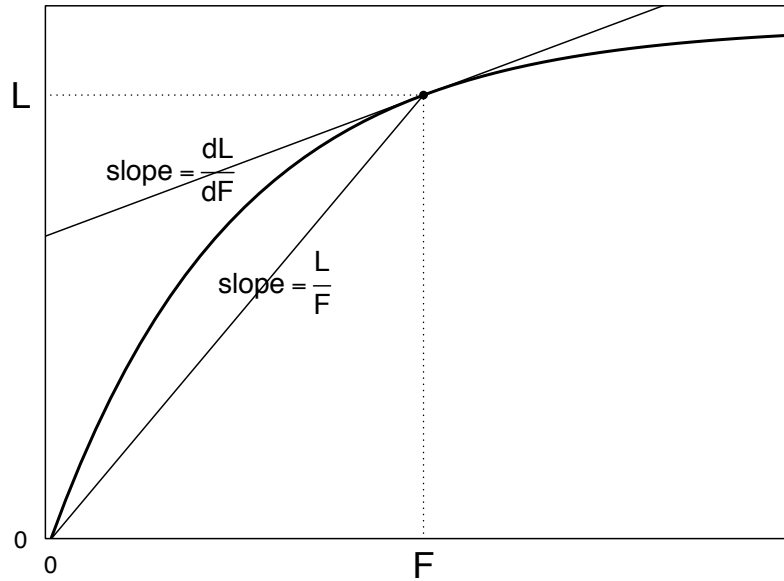


Figure 26: If the graph of L against F is concave-down, the slope dL/dF of the graph at any point will be less than the slope L/F of the line from the origin to the point, and from eq. (A.10) this makes $\partial D/\partial x$ negative. Note that if L were proportional to F , the graph would be linear and the two slopes would be the same making $\partial D/\partial x$ zero.

References

- Anderson, R. M. and R. M. May. 1991. Infectious diseases of humans: dynamics and control. Oxford University Press.
- Bliss, C. I. and R. A. Fisher. 1953. Fitting the negative binomial distribution to biological data. *Biometrics* 9: 176-200.
- Christiansen, F.B. 1991. On conditions for evolutionary stability for a continuously varying character, *American Naturalist* 138: 37-50.
- Clark, A. B. 1978. Sex ratio and local resource competition in a prosimian primate. *Science* 201: 163-165.
- Colwell, R. K. 1981. Group selection is implicated in the evolution of female-biased sex ratios. *Nature* 290: 401-404.
- Crofton, H. D. 1971. A quantitative approach to parasitism. *Parasitology* 62: 179-193.
- Edwards, A. W. F. 2000. Carl Düsing (1884) on the regulation of the sex-ratio. *Theoretical Population Biology* 58: 255-257.

- Fisher, R. A. 1930. The genetical theory of natural selection. Clarendon, Oxford.
- Hamilton, W. D. 1967. Extraordinary sex ratios. *Nature* 156: 477-488.
- Klein, T. A., D. G. Young, S. R. Telford, R. Kimsey. 1987. Experimental transmission of *Plasmodium mexicanum* by bites of infected *Lutzomyia vexator* (Diptera: Psychodidae). *Journal of the American Mosquito Control Association* 3: 154-164.
- Mackinnon, M. J. and A. F. Read. 2004. Virulence in malaria: an evolutionary viewpoint. *Transactions of the Royal Society of London Series B* 359: 965-986.
- Martinsen, E. M., S. L. Perkins and J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* 47: 261-273.
- Neal, A. T. 2011. Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 138: 1203-1210.
- Neal, A. T. and J. J. Schall. 2010. Gametocyte sex ratio in single-clone infections of the malaria parasite *Plasmodium mexicanum*. *Parasitology* 137: 1851-1859.
- Neal, A. T. and J. J. Schall. 2014a. Life history focus on a malaria parasite: linked traits and variation among genetic clones. *Evolutionary Ecology* 28: 89-102.
- Neal, A. T. and J. J. Schall. 2014b. Testing sex ratio theory with the malaria parasite *Plasmodium mexicanum* in natural and experimental infections. *Evolution* 68: 1071-1081.
- Osgood, S. M., R. J. Eisen and J. J. Schall. 2002. Gametocyte sex ratio of a malaria parasite: experimental test of heritability. *Journal of Parasitology* 88: 494-498.
- Osgood, S. M., R. J. Eisen, A. R. Wargo and J. J. Schall. 2003. Manipulation of the vertebrate host's testosterone does not affect gametocyte sex ratio of a malaria parasite. *Journal of Parasitology* 89: 190-192.
- Osgood, S. M. and J. J. Schall. 2004. Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* 128: 23-29.
- Paul, R. E. and K. P. Day 1998. Mating patterns of *Plasmodium falciparum*. *Parasitology Today* 14: 197-202.
- Pickering, J., A. F. Read, S. Guerrero, and S. A. West. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research* 2: 171-184.

- Read, A. F., M. Anwar, D. Shutler, and S. Nee. 1995. Sex allocation and population structure in malaria and related parasitic protozoa. *Proceedings of the Royal Society Series B* 260: 359-363.
- Read, A. F. and K. P. Day. 1992. The genetic structure of malaria parasite populations. *Parasitology Today* 8: 239-242.
- Read, A.F., A. Narara, S. Nee, A. K. Keymer, and K. P. Day. 1992. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Read, A. F. and L. H. Taylor. 2001. The ecology of genetically diverse infections. *Science* 292: 1099-1102.
- Reece, S. E., D. R. Drew and A. Gardner. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614.
- Reece, S. E., A. B. Duncan, S. A. West, and A. F. Read. 2003. Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*. *Parasitology* 127: 419-425.
- Robert, V. A. F. Read, J. Essong, T. Tchuinkam, B. Mulder, J.-P. Verhave, and P. Carnevale. 1996. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 621-624.
- Rosenberg, R. and J. Rungsiwongse. 1991. The number of sporozoites produced by individual malaria oocysts. *American Journal of Tropical Medicine and Hygiene* 45: 574-577.
- Schall, J. J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.
- Schall, J. J. 2009. Do malaria parasites follow the algebra of sex ratio theory? *Trends in Parasitology* 25: 120-123.
- Shutler, D., Bennett G. F. and Mullie, A. 1995. Sex proportions of *Haemoproteus* blood parasites and local mate competition. *Proceedings of the National Academy of Sciences* 92: 6748 – 6752.
- Shutler, D. and A. F. Read. 1998. Local mate competition, and extraordinary and ordinary blood parasite sex ratios. *Oikos* 82: 417-424.
- Sowunmi, A., S. T. Balogun, G. O. Gbotosho, and C. T. Happi. 2009a. *Plasmodium falciparum* gametocyte sex ratios in symptomatic children treated with antimalarial drugs. *Acta Tropica* 109: 108-117.

- Sowunmi, A., G. O. Gbotosho, C. T. Happi, O. A. Folarin, and S. T. Balogun. 2009b. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitology International* 58: 438-443.
- Taylor, L. H. 1997. Epidemiological and evolutionary consequences of mixed-genotype infections of malaria parasites. Ph. D. Dissertation, University of Edinburgh, UK.
- Taylor, L. H., D. Walliker and A. F. Read. 1997. Mixed-genotype infections of the rodent malaria parasite *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections. *Parasitology* 115: 121-132.
- Taylor, P. D. 1981. Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature* 291: 64-66.
- Taylor, P. D. and M. G. Bulmer. 1980. Local mate competition and the sex ratio. *Journal of Theoretical Biology* 86: 409-419.
- Trivers, R. L. and D. Willard. 1973. Natural selection of parental ability to vary the sex ratio of offspring. *Science* 179: 90-92.
- Ungureanu, E., R. Killick-Kendrick, P. C. C. Garnham, P. Branzei, C. Romanescu, P. G. Shute. 1976. Prepatent periods of a tropical strain of *Plasmodium vivax* after inoculations of tenfold dilutions of sporozoites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70: 482-483.
- Vardo-Zalik, A. M. 2009. Clonal diversity of a malaria parasite *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host. *International Journal for Parasitology* 39: 1573-1579.
- Vardo-Zalik, A. M. and J. J. Schall. 2009. Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* 90: 529-536.
- Vaughn, J. A. 2007. Population dynamics of *Plasmodium* sporogony. *Trends in Parasitology* 23: 63-70.
- West, S. A. 2009. Sex allocation. Princeton University Press, Princeton, NJ.
- West, S. A., S. E. Reece and A. F. Read. 2001. Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology* 17: 525-531.

West, S. A., T. G. Smith, S. Nee and A. F. Read. 2002. Fertility insurance and the sex ratio of malaria and related hemosporin blood parasites. *Journal of Parasitology* 88: 258-263.

Zollner, G. E., N. Ponsa, G. W. Garman, S. B. Poudel, J. A. Bell, J. Sattabongkot, R. E. Coleman, J. A. Vaughan. 2006. Population dynamics of sporogony for *Plasmodium vivax* parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. *Malaria Journal* 5: 68.

CHATER 8: Summary and Future Directions

Allison T. Neal

The original paper applying the Local Mate Competition (LMC) model to malaria parasites promised great utility: measuring a simple trait, the ratio of male to female parasites, could provide insight into parasite inbreeding rates for different host populations (Read et al. 1992). These data could put to rest debates concerning at what scale inbreeding should be measured (the parasite has to ‘know’ what scale is relevant; Nee et al. 2002) and provide an efficient, inexpensive method for measuring inbreeding (West 2009), which influences medically important phenomena like virulence evolution and the spread of drug and vaccine resistance (Babiker and Walliker 1997, Walliker et al. 1998, Read and Taylor 2001). However, subsequent tests of theory suggested a more complicated picture, with some studies failing to detect a relationship between measured or predicted selfing rates and sex ratios, and many additional studies suggesting that other factors, such as those implicated in Fertility Insurance, may further obscure the predicted relationship (see Chapter 1). This dissertation presents a series of studies that attempt to clarify the situation by providing appropriate tests of relevant predictions of LMC using a natural host-parasite association unaffected by antimalarial selective pressures. The results of this research suggest that while many predictions of LMC are supported qualitatively, a simple relationship between selfing and sex ratio is unlikely. This does not, however, imply that the study of malaria parasite sex ratios is not worthwhile. The insight gained from such studies may still have practical and theoretical importance, such

as in predicting responses to selective pressures imposed by treatment and the development of new theory and extension of existing models.

Summary of Results

Table 10 summarizes the major predictions tested in this dissertation and whether or not they were supported. The sex ratios of *Plasmodium mexicanum* appear to show heritable variation that is not constrained by the other life history traits measured (Chapters 2 and 6). Even in single-clone infections, these sex ratios are only slightly female-biased (Chapters 2 – 4 and 6), likely due to the limitation imposed by this species' apparent low male fecundity (Chapter 3). However despite the restrictions on the range of predicted sex ratios resulting from low male fecundity, sex ratio data from two separate experiments in which clonal diversity was experimentally manipulated showed evidence of facultative shifts in response to higher clonal diversity (lower selfing) that are qualitatively consistent with the predictions of LMC (Chapter 4). While this would seem to support the standard LMC with phenotypic plasticity model, these qualitative patterns are also consistent with extensions of LMC like fertility insurance and the newly proposed model (Chapter 7), which I will refer to as transmission bottleneck.

To date, fertility insurance has received more convincing support from empirical studies of malaria parasites than has standard LMC. It is therefore somewhat surprising that I found no evidence of fertility insurance in comparisons across infections of *P. mexicanum*. Associations between gametocytemia and sex ratio were either absent or positive (Chapters 2 – 6), contrary to the negative correlation predicted, and no consistent increase in sex ratio was observed over the course of infections (Chapter 2), as would be

predicted if parasites were responding to higher mortality from a building immune response. As noted in Chapter 4, the absence of these two patterns may be expected since *P. mexicanum* has high gametocytemia relative to other malaria parasites (Taylor and Read 1997), perhaps preventing mating group size from ever being small enough to warrant sex ratio adjustment, and there may be little immune-mediated mortality considering infections have never been known to be cleared (e.g. Bromwich and Schall 1986). As also discussed in more detail in Chapter 4, the absence of these predicted patterns within the species does not preclude the possibility that fertility insurance may play a role in the relatively unbiased sex ratios observed in this species overall.

Table 10: Summary of results from this dissertation. The organization of this table roughly corresponds to Figure # from the Chapter 1, with Transmission Biology replacing “LMC assumptions not met”. Note: the models are not mutually exclusive; for example, all models rely on sex ratios not being constrained, and Fertility Insurance and Transmission Bottlenecks are both extensions of LMC.

Model	Prediction	Supported?	Chapter
No Constraint	Sex ratios heritable	Yes	2, 6
No Constraint	No link to other traits	Yes	6
LMC- Phenotypic Plasticity	$r = 1/(1 + c)$ in single-clone infections	Yes	2, 3
LMC- Phenotypic Plasticity	$r = (n - 1)/2n$ across infections	Qualitatively	4
LMC- Local Adaptation	$r = (n - 1)/2n$ across sites	No, but n didn't vary	5
Fertility Insurance	r and gam negatively associated	No	2 – 6
Fertility Insurance	r increases over course of infection (with immune response)	No	2
Transmission Bottlenecks (Ch. 7)	r and n positively associated	Yes	4
Transmission Bottlenecks (Ch. 7)	r and gam positively associated	Mixed	Yes- 4, 5 No- 2, 3, 6
Transmission Bottlenecks (Ch. 7)	r and a positively associated	Suggestive	7

This dissertation also outlines a new extension to LMC related to the bottlenecks in transmission that likely limit fitness benefits associated with producing a female-biased sex ratio. The central prediction coming out of this model, that the strength of transmission bottlenecks should be positively associated with sex ratios, would be best tested across species. The little data that are currently available suggest a positive relationship (Chapter 7), but there are not currently enough data to make any conclusive claims. This is certainly an area that would benefit from further research. Other predictions, however, are more easily tested with data from a single species.

Transmission bottlenecks of the type modeled favor a positive association between clone number and sex ratio that is qualitatively similar to the prediction of standard LMC and consistent with the data presented in Chapter 4. Perhaps the strongest evidence for this new LMC extension, and evidence that current models are not sufficient, relates to the predicted positive relationship between the number of gametocytes ingested by the vector (related to gametocyte density and gametocytemia) and sex ratio. This pattern was observed at least bordering on significance ($P < 0.1$) in the data presented in both Chapters 4 and 5 of this dissertation, and has been described previously for *P. mexicanum* (Schall 2000) and other species (*Plasmodium tropiduri*: Pickering et al. 2000; *Plasmodium chabaudi*: Taylor 1997). This association is not predicted by any existing models in the absence of an indirect association via clone number, which was ruled out at least for *P. mexicanum* (Chapter 4) and *P. chabaudi* (Taylor 1997), and therefore highlights the potential utility of this new model in future research on sex ratio evolution in malaria parasites and other organisms.

Future Directions

What do the results of this dissertation suggest about the future of studies on malaria parasite sex ratios, malaria biology, and sex ratio theory? First, I believe it unlikely that sex ratios will live up to the original promise of a simple, inexpensive measure of absolute inbreeding rates. They may still reveal general patterns, which could be useful, but they are unlikely to resolve the debate over what scale of inbreeding is best. Understanding sex ratio evolution in malaria parasites has other potential practical uses, however, such as allowing us to predict the parasite's response to treatments that may have sex-specific effects (Ramiro et al. 2011), or identifying cues that could be used to artificially shift sex ratios toward those that might reduce transmission (Schall 2009). If the Transmission Bottleneck model proves valid, sex ratios may also be useful in predicting variation in malaria parasite transmission biology based on species or local conditions (e.g. parasite density in blood or regional insect vectors).

Second, this research suggests that certain methods of studying sex ratios in malaria parasites should be interpreted with caution. For example, prevalence may not reveal relevant patterns of variation in average clone numbers per infection. Parasite aggregation may vary with prevalence and the distribution of parasites may differ from the negative binomial, both of which can lead to very similar distributions of clones (and predicted sex ratios) among infections despite drastic differences in the proportion of hosts infected (Chapter 5). Also, even if more direct (e.g. genetic) estimates of clone numbers are employed, predicted patterns may not be detectable in natural infections due to confounding factors (Chapter 4). Co-infecting clones may have entered the infection at different times, making the genetic structure of the blood-stage parasites (trophozoites,

schizonts and immature and mature gametocytes) a poor indicator of the genetic structure of the mating population (mature gametocytes only). Clones found together naturally may also be closely related (due to multiple passages from insect to vertebrate and back) or tend to cooperate, which could alter sex ratio predictions (West 2009; Chapter 4). Additionally, clone numbers alone may not be sufficient to predict sex ratio: it may be necessary to also incorporate relative proportions of clones with a measurement akin to clonal diversity (Chapter 4). Finally, male fecundity may also limit the range in clonal diversity over which an association with sex ratio is predicted, which could be misleading if not accounted for (Chapters 3, 4).

The data presented in this dissertation also have many heartening aspects. In the study of malaria parasite biology, this research provides further strength to the growing body of evidence suggesting that malaria parasites are able to recognize the presence of unrelated parasite strains and adjust their sex ratios (or presumably other life history traits, etc.) accordingly (Reece et al. 2008, Sowunmi et al. 2009, Chapter 4). It also provides further evidence of heritable sex ratio variation for malaria parasites (Chapters 2 and 6) and one of the first thorough quantifications of male gamete production (Chapter 3). Further, Chapter 7 of this dissertation highlights the importance of transmission and the need for more detailed research on patterns of transmission, particularly from the insect to the vertebrate. Very few data exist on how many parasites are injected into a vertebrate host, to what extent this number is influenced by oocyst or sporozoite numbers, and whether competition exists for sporozoite production speed or localization in the salivary glands. If the new Transmission Bottleneck model is supported, sex ratio

research also has the potential to contribute to our understanding of this relatively overlooked area.

Finally, the Transmission Bottleneck model also expands the explanatory power of sex ratio theory. While the general selective forces underlying LMC appear to be influential in shaping malaria parasite sex ratios and studies of malaria parasites like the research presented in this dissertation broaden the taxonomic diversity for which this evolutionary theory has been tested, an examination of the basic life cycle of malaria parasites coupled with the positive association between gametocytemia and sex ratio observed for multiple *Plasmodium* species suggest that existing models are not sufficient to explain the patterns observed. This new model therefore presents a potentially influential new direction for sex ratio research in malaria parasites and beyond. While transmission bottlenecks sound particularly suited to parasitic organisms, they could equally be interpreted as anything restricting the passage of genes from one generation to the next, such as predation and competition. If these forces are density dependent such that the per capita transmission of genes decreases (e.g. from increased per capita mortality or decreased per capita fecundity) as the number of mated females increases, this new model may help explain sex ratio evolution.

Concluding statements

Even though the origins of sex ratio theory and the recognition of distinct male and female cells in malaria parasites can both be traced to just around the turn of the 20th century, only in the past 25 years have attempts been made to merge the study of sex ratios and malaria parasites (Read et al. 1992, Schall 1989, Schall 2009). Prior tests of

theoretical predictions yielded controversial results, highlighting gaps and insufficiencies in our understanding of underlying processes and theory. Through the use of genetic markers to test both theoretical predictions and assumptions, the employment of light microscopy to estimate male fecundity (not previously done rigorously), and the development of new theoretical models, the work in this dissertation has helped to fill some of these gaps, provide examples of useful methods and areas for future research, and push the field further both empirically and theoretically.

References

- Babiker, H. A. and D. Walliker. 1997. Current views on the population structure of *Plasmodium falciparum*: implications for control. *Parasitol. Today* 13: 262-267.
- Bromwich, C. R. and J. J. Schall. 1986. Infection dynamics of *Plasmodium mexicanum*, a malaria parasite of lizards. *Ecology* 67: 1227-1235.
- Nee, S., S. A. West and A. F. Read. 2002. Inbreeding and parasite sex ratios. *Proc. Roy. Soc. Lond. B* 269: 755-760.
- Pickering, J., A. F. Read, S. Guerrero, and S. A. West. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evol. Ecol. Res.* 2: 171-184.
- Ramiro, R. S., J. Alpedrinha, L. Carter, A. Gardner, and S. E. Reece. 2011. Sex and death: the effects of the innate immune factors on the sexual reproduction of malaria parasites. *PLoS Pathogens* 3: e1001309.
- Read, A. F., A. Narara, S. Nee, A. K. Keymer, and K. P. Day. 1992. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Read, A. F. and L. H. Taylor. 2001. The ecology of genetically diverse infections. *Science* 292: 1099-1102.
- Reece, S. E., D. R. Drew and A. Gardner. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614.
- Taylor, L. H. 1997. Epidemiological and evolutionary consequences of mixed-genotype infections of malaria parasites. Ph. D. Dissertation, University of Edinburgh, UK.

Taylor, L. H. and A. F. Read. 1997. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today* 13: 135-140.

Schall, J. J. 1989. The sex ratio of *Plasmodium* gametocytes. *Parasitology* 98: 343-350.

Schall, J. J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.

Schall, J. J. 2009. Do malaria parasites follow the algebra of sex ratio theory? *Trends Parasitol.* 25: 120-123.

Sowunmi, A., G. O. Gbotosho, C. T. Happi, O. A. Folarin, and S. T. Balogun. 2009. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitol. Int.* 58: 438-443.

Walliker, D., H. Babiker and L. Ranford-Cartwright. 1998. The genetic structure of malaria parasite populations. In: Sherwin, I. W. (Ed.). *Malaria: Parasite Biology, Pathogenesis and Protection* (pp. 235-252) ASM Press, Washington DC.

West, S. 2009. Sex allocation. Princeton University Press, Princeton.

COMPREHENSIVE BIBLIOGRAPHY

- Alano, P. and O. Billker. 2005. Gametocytes and Gametes. In: Sherman, I. W. (Ed.) *Molecular Approaches to Malaria* (pp. 191-219). ASM Press, Washington DC.
- Alano, P. and R. Carter. 1990. Sexual differentiation in malaria parasites. *Annual Reviews of Microbiology* 44: 429-449.
- Al-Olayan, E. M., G. T. Williams and H. Hurd. 2002. Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *International Journal of Parasitology* 32: 1133-1143.
- Anderson T. J., B. Haubold, J. T. Williams, J. G. Estrada-Franco, L. Richardson, R. Mollinedo, M. Bockarie, J. Mokili, S. Mharakurwa, N. French, J. Whitworth, I. D. Velez, A. H. Brockman, F. Nosten, M. U. Ferreira and K. P. Day. 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Molecular Biology and Evolution* 17: 1467-1482.
- Anderson, R. M. and R. M. May. 1991. *Infectious Diseases of Humans: Dynamics and Control*. Oxford University Press, New York.
- Anderson, T. J., R. E. Paul, C. A. Donnelly and K. P. Day. 2000. Do malaria parasites mate non-randomly in the mosquito midgut? *Genetical Research* 75: 285-296.
- Anderson, T. J. C., J. T. Williams, S. Nair, D. Sudimack, M. Barends, A. Jaidee, R. N. Price and F. Nosten. 2010. Inferred relatedness and heritability in malaria parasites. *Proceedings of the Royal Society Series B: Biology* 277: 2531-2540.
- Babiker, H. A. and D. Walliker (1997) Current views on the population structure of *Plasmodium falciparum*: implications for control. *Parasitology Today* 13: 262-267.
- Baker, D. A. 2010. Malaria gametocytogenesis. *Molecular and Biochemical Parasitology* 172: 57-65.
- Barclay, V. C., D. Sim, B. H. K. Chan, L. A. Nell, M. A. Rabaa, A. S. Bell, R. F. Anders and A. F. Read. 2012. The evolutionary consequences of blood-stage vaccination on the rodent malaria *Plasmodium chabaudi*. *PLoS Biology* 10: e1001368.
- Bliss, C. I. and R. A. Fisher. 1953. Fitting the negative binomial distribution to biological data. *Biometrics* 9: 176-200.
- Boudin, C., M. Olivier, J. F. Molez, J. P. Chiron and P. Ambroise-Thomas. 1993. High human malarial infectivity to laboratory bred *Anopheles gambiae* in a village in Burkina Faso. *American Journal of Tropical Medicine and Hygiene* 48: 700-706.

- Bromwich, C. R. and J. J. Schall. 1986. Infection dynamics of *Plasmodium mexicanum*, a malaria parasite of lizards. *Ecology* 67: 1227-1235.
- Buckling, A. G., L. Crooks and A. F. Read. 1999. *Plasmodium chabaudi*: effect of antimalarial drugs on gametocytogenesis. *Experimental Parasitology* 93: 45-54.
- Buckling, A. G., L. H. Taylor, J. M.-R. Carlton and A. F. Read. 1997. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proceedings of the Royal Society of London Series B: Biology* 264: 553-559.
- Bull, J. J. and E. L. Charnov. 1988. How fundamental are Fisherian sex ratios? *Oxford Surveys in Evolutionary Biology* 5: 96-135.
- Bulmer, M. G. and P. D. Taylor. 1980. Sex ratio under the haystack model. *Journal of Theoretical Biology* 86: 83-89.
- Burkot, T. R., J. L. Williams and I. Schneider. 1984. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown in vitro from the same isolate. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 78: 339-341.
- Carter, R. and P. M. Graves. 1988. Gametocytes. In: Wernsdorfer, W. H. and I. McGregor (Ed.). *Malaria: Principles and Practice of Malariology* (pp. 253-305). Churchill Livingstone, Edinburgh, UK.
- Carter, R., R. W. Gwadz and F. M. McAuliffe. 1979. *Plasmodium gallinaceum*: Transmission-blocking immunity in chickens. I. Comparative immunogenicity of gametocyte- and gamete-containing preparations. *Experimental Parasitology* 47: 185-193.
- Carter R. and L. H. Miller. 1979. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bulletin of the World Health Organization* 57 S1: 37-52.
- Charnov, E. L. 1982. *The Theory of Sex Allocation*. Princeton University Press, Princeton. *Monographs in Population Biology* 18.
- Christiansen, F.B. 1991. On conditions for evolutionary stability for a continuously varying character, *American Naturalist* 138: 37-50.
- Clark, A. B. 1978. Sex ratio and local resource competition in a prosimian primate. *Science* 201: 163-165.
- Colwell, R. K. 1981. Group selection is implicated in the evolution of female-biased sex ratios. *Nature* 290: 401-404.

Crofton, H. D. 1971. A quantitative approach to parasitism. *Parasitology* 62: 179-193.

Darwin, C. 1871. *The descent of man, and selection in relation to sex*. John Murray, London, U.K.

de Roode, J. C., M. E. H. Helinski, M. A. Anwar and A. F. Read. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *American Naturalist* 166: 531-542.

Dunlap K. D. and J. J. Schall. 1995. Hormonal alterations and reproductive inhibition in male fence lizards (*Sceloporus occidentalis*) infected with the malaria parasite *Plasmodium mexicanum*. *Physiological Zoology* 68: 608-621.

Düsing, C. 1884. *Die regulierung des geschlechtsverhältnisses bei der vermehrung der menschen, tiere und pflanzen*. Fischer, Jena.

Dye, C. and H. C. F. Godfray. 1993. On sex ratio and inbreeding in malaria parasite populations. *Journal of Theoretical Biology* 161: 131-134.

Edwards, A. W. F. 1998. Natural selection and the sex ratio: Fisher's sources. *American Naturalist* 151: 564-569.

Edwards, A. W. F. 2000. Carl Düsing (1884) on the regulation of the sex-ratio. *Theoretical Population Biology* 58: 255-257.

Eisen, R. J. 2000. Variation in life-history traits of *Plasmodium mexicanum*, a malaria parasite infecting western fence lizards: a longitudinal study. *Canadian Journal of Zoology* 78: 1230-1237.

Eisen, R. J. and J. J. Schall. 2000. Life history of a malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *Proceedings of the Royal Society of London* 267: 793-799.

Eisen, R.J. and N. M. Wright. 2001. Landscape features associated with infection by a malaria parasite (*Plasmodium mexicanum*) and the importance of multiple scale studies. *Parasitology* 122: 507-513.

Fialho, R. F. and J. J. Schall. 1995. Thermal ecology of a malarial parasite and its insect vector: Consequences for the parasite's transmission success. *Journal of Animal Ecology* 64: 553-562.

Fisher, R. A. 1930. *The Genetical Theory of Natural Selection*. Dover Publications, New York.

Ford, A. F. and J. J. Schall. 2011. Relative clonal proportions over time in mixed-genotype infections of the lizard malaria parasite *P. mexicanum*. *International Journal of Parasitology* 41: 731-738.

Ford, A. F., A. M. Vardo-Zalik, and J. J. Schall. 2010. Relative clonal density of malaria parasites in mixed-genotype infections: validation of a technique using microsatellite markers for *Plasmodium falciparum* and *P. mexicanum*. *Journal of Parasitology* 96: 908-913.

Frank, S. A. 1986. Hierarchical selection theory and sex ratios. I. General solutions for structured populations. *Theoretical Population Biology* 29: 312-342.

Fricke, J. M., A. M. Vardo-Zalik and J. J. Schall. 2010. Geographic genetic differentiation of a malaria parasite, *Plasmodium mexicanum*, and its lizard host, *Sceloporus occidentalis*. *Journal of Parasitology* 96: 308-313.

Gardner, A., S. Reece. S. West. 2003. Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *Journal of Theoretical Biology* 223: 515-521.

Garnham, P. C. C. 1966. *Malaria parasites and Other Hemosporidia*. Blackwell Scientific Publications, Oxford, UK

Gatei, W., S. Kariuki, W. Hawley, F. ter Kuile, D. Terlouw, P. Phillips-Howard, B. Nahlen, J. Gimnig, K. Lindblade, E. Walker, M. Hamel, S. Crawford, J. Williamson, L. Slutsker and Y. P. Shi. 2010. Effects of transmission reduction by insecticide-treated bed nets (ITNs) on parasite genetics population structure: I. The genetic diversity of *Plasmodium falciparum* parasites by microsatellite markers in western Kenya. *Malaria Journal* 9: 353.

Gaudart, J., B. Poudiougou, A. Dicko, S. Ranque, O. Toure, I. Sagara, M. Diallo, S. Diawara, A. Ouattara, M. Diakite and O. K. Doumbo. 2006. Space-time clustering of childhood malaria at the household level: a dynamic cohort in a Mali village. *BMC Public Health* 6: 286.

Ghiselin, M. T. 1974. *The Economy of Nature and the Evolution of Sex*. University of California Press, Berkeley.

Godfray, H. C. J. and J. H. Werren. 1996. Recent developments in sex ratio studies. *Trends in Ecology and Evolution* 11: 59-63.

Goldstein, D. B. and C. Schlotterer (Ed.) 1999. *Microsatellites: Evolution and Applications*. Oxford University Press, Oxford.

Gotelli, N. J. 2008. *A Primer of Ecology*. 4th ed. Sinauer Associates, Sunderland, MA.

- Gotelli, N. J. and R. K. Colwell. 2009. Estimating species richness. In: Magurran, A. E. and B. J. MacGill (Ed.) *Biological Diversity: Frontiers in Measurement and Assessment* (pp 39-54). Oxford University Press, Oxford.
- Gotelli, N. J. and A. M. Ellison. 2004. *A Primer of Ecological Statistics*. 1st ed. Sinauer Associates, Sunderland, MA.
- Graves, P. M., R. Carter and K. M. McNeill. 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* 33: 1045-1050.
- Guilbride, D. L., P. D. L. Guilbride and P. Gawlinski. 2012. Malaria's deadly secret: a skin stage. *Trends in Parasitology* 4: 142-150.
- Hartl, D. L. 1971. Some aspects of natural selection in arrhenotokous populations. *American Zoologist* 11: 309-325.
- Hamilton, W. D. 1967. Extraordinary sex ratios. *Science* 156: 477-488.
- Herbers, J. M. 1984. Queen-worker conflict and eusocial evolution in a polygynous ant species. *Evolution* 38: 631-643.
- Hoaglin, D. C., F. Mosteller and J. W. Tukey. 1983. Introduction to more refined estimators. In: Hoaglin, D. C., F. Mosteller and J. W. Tukey (Ed.) *Understanding Robust and Exploratory Data Analysis* (pp. 283-296). John Wiley & Sons, Inc., New York, USA.
- James, S. P. 1931. Some general results of a study of induced malaria in England. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 24: 477-538.
- Joliffe, I. T. 2002. *Principal component analysis*: Second edn. Springer, New York.
- Klein, T. A., D. G. Young, S. R. Telford, R. Kimsey. 1987. Experimental transmission of *Plasmodium mexicanum* by bites of infected *Lutzomyia vexator* (Diptera: Psychodidae). *Journal of the American Mosquito Control Association* 3: 154-164.
- Liu, Z., J. Miao and L. Cui. 2011. Gametocytogenesis in malaria parasite: commitment, development and regulation. *Future Microbiology* 6: 1351-1369.
- Lobo, C. A. and N. Kumar. 1998. Sexual differentiation and development in the malaria parasite. *Parasitology Today* 14: 146-150.
- Mara, S. E., K. D. Silué, G. Raso, S. P. N'guetta, E. K. N'goran, M. Tanner, J. Utzinger and X. C. Ding. 2013. Genetic diversity of *Plasmodium falciparum* among school-aged children from the Man region, western Côte d'Ivoire. *Malaria Journal* 12: 419.

- Martinsen, E. S., S. L. Perkins, J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* 47: 261-273.
- Mackinnon, M. J. and A. F. Read. 1999. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* 53: 689-703.
- Mackinnon M. J. and A. F. Read. 1999. Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. *Proceedings of the Royal Society of London Series B: Biology* 266: 741-748.
- Maynard Smith, J. 1982. *Evolution and the Theory of Games*. Cambridge University Press, New York.
- Merino, S., G. Tomás, J. Moreno, J. J. Sanz, E. Arriero and C. Folgueira. 2004. Changes in *Haemoproteus* sex ratios: fertility insurance or differential sex lifespan? *Proceedings of the Royal Society of London Series B: Biology* 271: 1605-1609.
- Mideo, N. and T. Day. 2008. On the evolution of reproductive restraint in malaria. *Proceedings of the Royal Society of London Series B: Biology* 275: 1217-1224.
- Mitri, C., I. Thiery, C. Bourgouin and R. E. L. Paul. 2009. Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates. *Proceedings of the Royal Society of London Series B: Biology* 276: 3721-3726.
- Mousseau, T. A. and D. A. Roff. 1987. Natural selection and the heritability of fitness components. *Heredity* 59: 181-197.
- Murray, C. J. L., R. C. Rosenfeld, S. S. Lim, K. G. Andrews, K. J. Foreman, D. Haring, N. Fullman, M. Naghavi, R. Lozano and A. D. Lopez. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet* 379: 413-431.
- Neal, A. T. 2011. Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 138: 1203-1210.
- Neal, A. T. and J. J. Schall. 2010. Gametocyte sex ratio in single clone infections of the malaria parasite *Plasmodium mexicanum*. *Parasitology* 137: 1851-1859.
- Neal A. T. and J. J. Schall. 2014. Testing sex ratio theory with the malaria parasite *Plasmodium mexicanum* in natural and experimental infections. *Evolution* 68: 1071-1081.
- Neal, A. T. and J. J. Schall. 2014. Life history focus on a malaria parasite: linked traits and variation among genetic clones. *Evolutionary Ecology* 28: 89-102.

Neal, A.T. and P. D. Taylor. Local mate competition and transmission bottlenecks: a new model for understanding malaria parasite and other sex ratios. *Journal of Theoretical Biology* (in review)

Nee, S., S. A. West and A. F. Read. 2002. Inbreeding and parasite sex ratios. *Proceedings of the Royal Society of London Series B: Biology* 269: 755-760.

Orzack, S. H. 2002. Using sex ratios: the past and the future. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 383-398). Cambridge University Press, New York.

Osgood, S. M., R. J. Eisen and J. J. Schall. 2002. Gametocyte sex ratio of a malaria parasite: experimental test of heritability. *Journal of Parasitology* 88: 494-498.

Osgood, S. M., R. J. Eisen, A. R. Wargo and J. J. Schall. 2003. Manipulation of the vertebrate host's testosterone does not affect gametocyte sex ratio of a malaria parasite. *Journal of Parasitology* 89: 190-192.

Osgood, S. M. and J. J. Schall. 2004. Gametocyte sex ratio of a malaria parasite: response to experimental manipulation of parasite clonal diversity. *Parasitology* 128: 23-29.

Paperna, I. and I. Landau. 1991. *Haemoproteus* (Haemosporidia) of lizards. *Bulletin du Museum National d'Histoire Naturelle Section A, Zoologie, Biologie et Ecologie Animales* 13: 309-349.

Paul, R. E. 2000. Sex determination in malaria parasites. *Science* 287: 128-131.

Paul, R. E. L., P. T. Brey and V. Robert. 2002. *Plasmodium* sex determination and transmission to mosquitoes. *Trends in Parasitology* 18: 32-38.

Paul, R. E. and K. P. Day. 1998. Mating patterns of *Plasmodium falciparum*. *Parasitology Today* 14: 197-202.

Paul, R. E., M. J. Packer, M. Walmsley, M. Lagog, L. C. Ranford-Cartwright, R. Paru and K. P. Day. 1995. Mating patterns in malaria populations of Papua New Guinea. *Science* 269: 1709-1711.

Paul, R. E. L., A. Raibaud and P. T. Brey. 1999. Sex ratio adjustment in *Plasmodium gallinaceum*. *Parassitologia* 41: 153-158.

Pen, I. and F. J. Weissing. 2002. Optimal sex allocation: steps towards a mechanistic theory. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 26-45). Cambridge University Press, New York.

- Perkins, S. L., S. M. Osgood and J. J. Schall. 1998. Use of PCR for detection of subpatent infections of lizard malaria: implications for epizootiology. *Molecular Ecology* 7: 1587-1590.
- Pickering, J., A. F. Read, S. Guerrero and S. A. West. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research* 2: 171-184.
- Pollitt, L. C., N. Mideo, D. R. Drew, P. Schneider, N. Colegrave and S. E. Reece. 2011. Competition and evolution of reproductive restraint in malaria parasites. *American Naturalist* 177: 358-367.
- Ramiro, R. S., J. Alpedrinha, L. Carter, A. Gardner, and S. E. Reece. 2011. Sex and death: the effects of the innate immune factors on the sexual reproduction of malaria parasites. *PLoS Pathogens* 3: e1001309.
- Ranford-Cartwright, L. C. 1995. Fit for fertilization: mating in malaria parasites. *Parasitology Today* 11: 154-157.
- Ranford-Cartwright, L. C., P. Balfe, R. Carter and D. Walliker. 1993. Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology* 107: 11-18.
- Read, A. F., M. Anwar, D. Shutler and S. Nee. 1995. Sex allocation and population structure in malaria and related parasitic protozoa. *Proceedings of the Royal Society of London Series B: Biology* 260: 359-363.
- Read, A. F. and K. P. Day. 1992. The genetic structure of malaria parasite populations. *Parasitology Today* 8: 239-242.
- Read, A. F., A. Narara, S. Nee, A. K. Keymer, and K. P. Day. 1992. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.
- Read, A. F., T. G. Smith, S. Nee and S. A. West. 2002. Sex ratios of malaria parasites and related protozoa. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 314-332). Cambridge University Press, New York.
- Read, A. F. and L. H. Taylor. 2001. The ecology of genetically diverse infections. *Science* 292: 1099-1102.
- Reece, S. E., D. R. Drew and A. Gardner. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614.
- Reece, S. E., A. B. Duncan, S. A. West and A. F. Read. 2003. Sex ratios in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* 127: 519-525.

- Reece, S. E., R. S. Ramiro and D. H. Nussey. 2009. Plastic parasites: sophisticated strategies for survival and reproduction. *Evolutionary Applications* 1: 11-23.
- Reznick, D. and J. A. Endler. 1982. The impact of predation on life history evolution in the Trinidadian guppies (*Poecilia reticulata*). *Evolution* 36: 160-177.
- Robert, V., A. F. Read, J. Essong, T. Tchuinkam, B. Mulder, J.-P. Verhave and P. Carnevale. 1996. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 621-624.
- Robert, V., C. S. Sokhna, C. Rogier, F. Ariey, and J.-F. Trape. 2003. Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* 127: 1-8.
- Rosenberg, R. and J. Rungsiwongse. 1991. The number of sporozoites produced by individual malaria oocysts. *American Journal of Tropical Medicine and Hygiene* 45: 574-577.
- Schall, J. J. 1989. The sex ratio of *Plasmodium* gametocytes. *Parasitology* 98: 343-350.
- Schall, J. J. 1990. Virulence of lizard malaria: The evolutionary ecology of an ancient parasite-host association. *Parasitology* 100: S35-S52.
- Schall, J. J. 1996. Malarial parasites of lizards: diversity and ecology. *Advances in Parasitology* 37: 255-333.
- Schall, J. J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.
- Schall, J. J. 2002. Parasite virulence. In: Lewis, E., J. Campbell and M. Sukhdeo (Ed.) *The Behavioural Ecology of Parasites* (pp. 283-313). CAB International, Oxon, UK.
- Schall, J. J. 2008. Sex ratios writ small. *Nature* 453: 605-606.
- Schall, J. J. 2009. Do malaria parasites follow the algebra of sex ratio theory? *Trends in Parasitology* 25: 120-123.
- Schall, J. J., A. Bennett and R. Putnam. 1982. Lizards infected with malaria: physiological and behavioral consequences. *Science* 217: 1057-1058.
- Schall, J. J. and A. B. Marghoob. 1995. Prevalence of a malaria parasite over time and space: *Plasmodium mexicanum* in its vertebrate host, the western fence lizard *Sceloporus occidentalis*. *Journal of Animal Ecology* 64: 177-185.

Schall, J. J. and K. M. St. Denis. 2013. Microsatellite loci over a thirty-three year period for a malaria parasite (*Plasmodium mexicanum*): bottleneck in effective population size and effect on allele frequencies. *Parasitology* 140: 21-28.

Schall, J. J. and A. M. Vardo. 2007. Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes. *Molecular Ecology Notes* 7: 227-229.

Shutler, D., G. F. Bennett and A. Mullie. 1995. Sex proportions of *Haemoproteus* blood parasites and local mate competition. *Proceedings of the National Academy of Sciences* 92: 6748-6752.

Shutler, D. and A. F. Read. 1998. Local mate competition, and extraordinary and ordinary blood parasite sex ratios. *Oikos* 82: 417-424.

Sinden, R. E. 1983. Sexual development of malaria parasites. *Advances in Parasitology* 22: 154-216.

Sinden, R. E., E. U. Canning, R. S. Bray and M. E. Smalley. 1978. Gametocyte and gamete development in *Plasmodium falciparum*. *Proceedings of the Royal Society of London Series B: Biology* 201: 375-399.

Seeger, J. and J. W. Stubblefield. 2002. Models of sex ratio evolution. In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 2-25). Cambridge University Press, New York.

Stearns, S. C. 1992. *The evolution of life histories*. Oxford University Press, New York.

Smalley, M. E. and R. E. Sinden. 1977. *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* 74: 1-8.

Sober, E. 2001. Sex ratio theory, ancient and modern - an 18 century debate about intelligent design and the development of models in evolutionary biology. In: Riskin, J. (Ed.) *Genesis Redux: Essays in the History and Philosophy of Artificial Life* (pp. 131-162) The University of Chicago Press, Chicago.

Sowunmi, A., S. T. Balogun, G. O. Gbotosho and C. T. Happi. 2009a. *Plasmodium falciparum* gametocyte sex ratios in symptomatic children treated with antimalarial drugs. *Acta tropica* 109: 108-117.

Sowunmi, A., G. O. Gbotosho, C. T. Happi, O. A. Folarin, and S. T. Balogun. 2009b. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitology International* 58: 438-443.

Stubblefield, J. W. and J. Seger. 1990. Local mate competition with variable fecundity: dependence of offspring sex ratios on information utilization and mode of male production. *Behavioral Ecology* 1: 68-80.

Taylor, L. H. 1997. Epidemiological and evolutionary consequences of mixed-genotype infections of malaria parasites. Ph. D. Dissertation, University of Edinburgh, UK.

Taylor, L. H. and A. F. Read. 1997. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitology Today* 13: 135-140.

Taylor, L. H., D. Walliker and A. F. Read. 1997 Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. *Proceedings of the Royal Society of London Series B: Biology* 264: 927-935.

Taylor, P. D. 1981. Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature* 291: 64-66.

Taylor, P. D. and M. Bulmer. 1980. Local mate competition and the sex ratio. *Journal of Theoretical Biology* 86: 409-419.

Trager, W., M. Tershakovec, L. Lyandvert, H. Stanley, N. Lanners and E. Gubert. 1981. Clones of the malaria parasite *Plasmodium falciparum* obtained by microscopic selection: their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes. *Proceedings of the National Academy of Science* 78: 6527-6530.

Trivers, R. L. 1974. Parent-offspring conflict. *American Zoologist* 14: 249-264.

Trivers, R. L. and D. E. Willard. 1973. Natural selection of parental ability to vary the sex ratio of offspring. *Science* 179: 90-92.

Ungureanu, E., R. Killick-Kendrick, P. C. C. Garnham, P. Branzei, C. Romanescu, P. G. Shute. 1976. Prepatent periods of a tropical strain of *Plasmodium vivax* after inoculations of tenfold dilutions of sporozoites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70: 482-483.

Van Noordwijk, A. J. and G. De Jong. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist* 128: 137-142.

Vardo, A. M. and J. J. Schall. 2007. Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Molecular Ecology* 16: 2712-2720.

Vardo, A. M., K. D. Kaufhold and J. J. Schall. 2007. Experimental test for premunition in a lizard malaria parasite (*Plasmodium mexicanum*). *Journal of Parasitology* 93: 280-282.

- Vardo-Zalik, A. M. 2009. Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host. *International Journal of Parasitology* 39: 1573-1579.
- Vardo-Zalik, A. M., A. F. Ford, and J. J. Schall. 2009. Detecting number of clones, and their relative abundance, of a malaria parasite (*Plasmodium mexicanum*) infecting its vertebrate host. *Parasitology Research* 105: 209-215.
- Vardo-Zalik, A. M. and J. J. Schall. 2008. Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*. *Parasitology* 135: 1363-1372.
- Vardo-Zalik, A. M. and J. J. Schall. 2009. Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host. *Ecology* 90: 529-536.
- Vaughn, J. A. 2007. Population dynamics of *Plasmodium* sporogony. *Trends in Parasitology* 23: 63-70.
- Walliker, D., H. Babiker and L. Ranford-Cartwright. 1998. The genetic structure of malaria parasite populations. In: Sherwin, I. W. (Ed.). *Malaria: Parasite Biology, Pathogenesis and Protection* (pp. 235-252) ASM Press, Washington DC.
- West, S. 2009. *Sex allocation*. Princeton University Press, Princeton.
- West, S. A. and E. A. Herre. 2002. Using sex ratios: why bother? In: Hardy, I. C. W. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 399-413). Cambridge University Press, New York.
- West, S. A., S. E. Reece and A. F. Read. 2001. Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology* 17: 525-531.
- West, S. A., T. G. Smith, S. Nee and A. F. Read. 2002. Fertility insurance and the sex ratios of malaria and related Hemospororin blood parasites. *Journal of Parasitology* 88: 258-263.
- Williams, G. C. 1966. *Adaptation and Natural Selection*. Princeton University Press, Princeton.
- Wilson, D. S. and R. K. Colwell. 1981. Evolution of sex ratios in structured demes. *Evolution* 35: 882-897.
- Wilson, K. and I. C. W. Hardy. 2002. Statistical analysis of sex ratios: an introduction. In: Hardy, I. W. C. (Ed.) *Sex Ratios: Concepts and Research Methods* (pp. 48-92). Cambridge University Press, Cambridge.

Yamaguchi, Y. 1985. Sex ratios of an aphid subject to local mate competition with variable maternal condition. *Nature* 318: 460-462.

Zhong, D., Y. Afrane, A. Githeko, Z. Yang, L. Cui, D. M. Menge, E. A. Temu and G. Yan. 2007 *Plasmodium falciparum* genetic diversity in western Kenya highlands. *American Journal of Tropical Medicine and Hygiene* 77: 1043-1050.

Zollner, G. E., N. Ponsa, G. W. Garman, S. Poudel, J. A. Bell, J. Sattabongkot, R. E. Coleman and J. A. Vaughan. 2006. Population dynamics of sporogony for *Plasmodium vivax* parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. *Malaria Journal* 5: 68.